THE UNIVERSITY
of EDINBURGH

THE EFFECT OF ORAL HYPOGLYCAEMIC AGENTS ON PLACENTAL ABC TRANSPORTER EXPRESSION AND ACTIVITY

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MScR

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2016

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Submission: January 2016

I confirm the following:

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14th July 2016

Abstract

Gestational Diabetes Mellitus (GDM) affects between 3-10% of pregnancies with consequences for both the mother and the newborn infant. The target of GDM management is achieving euglycaemia as this has been demonstrated to reduce adverse neonatal events.

With the increasing incidence of GDM there is a drive to manage this condition in not only the safest way possible but also in a way which is acceptable to women, with the easiest route of administration of medication and dosing schedule to increase their concordance. Therefore, attention has turned to looking at the use of oral hypoglycaemic agents (OHAs) for the treatment of GDM. There are two classes of OHAs used in pregnancy: biguanides (for example metformin) and sulphonylureas (for example glibenclamide, also known as glyburide).

In the placenta a network of apical ATP-binding cassette (ABC) transporters facilitates the efflux of substances away from the fetus, thereby protecting the fetus from exposure to unwanted substances including medications such as metformin and glibenclamide. In this thesis, I have examined the mRNA levels of P-glycoprotein (P-gp), Breast cancer resistance protein (BCRP) and Multidrug resistance-associated protein (MRP) in placentas from women with GDM. I have demonstrated a reduction in P-gp mRNA expression among placentas from those women treated with metformin and insulin compared to placentas from women without GDM and those treated with metformin alone. *In vitro* treatment of placental explants with metformin and glibenclamide demonstrated no effect upon the mRNA levels of P-gp, BCRP, MRP and Glucose 1 transporter (GLUT 1).

While treatment with OHAs is becoming standard care in the management of GDM, little work has been done looking at those mechanisms in place to protect the fetus from such treatments. Future developments in the management of GDM are likely to include combining metformin and glibenclamide; the work in this thesis provides *in vitro* evidence that this combined treatment does not alter the ABC transporters protecting the fetus and therefore supports future clinical trials for such a treatment.

Lay Summary

Up to one in ten pregnant women develop diabetes in pregnancy, known as Gestational Diabetes. These women and their unborn babies are at an increased risk of pregnancy complications. It is known that treating these women with medications during pregnancy can reduce these risks. The traditionally accepted treatment was with insulin injections, however; more recently these women have been treated with two types of tablets with good effect.

Whilst there have been several studies showing that these tablets are effective at treating the gestational diabetes with few side effects for the babies, there is little evidence regarding the transport of these medications to the baby across the placenta during the pregnancy.

In the placenta (which connects the baby to the mother and is responsible for passing substances such as oxygen and nutrients to the baby and removing unwanted waste products from the baby) there are important systems in place to transport substances both towards and away from the baby (including transporter proteins). It is known that some of these transporter proteins protect the baby from exposure to unwanted levels of the medications used in treating gestational diabetes.

In this report we have looked at these transporter proteins in placentas from women who had gestational diabetes during their pregnancies and compared them to placentas from women who did not have diabetes in pregnancy. We have shown that in general there was no difference in their levels except among those placentas from women with diabetes who were treated with tablets and insulin injections; there were lower levels of one of the transporter proteins in this group of placentas compared to

those from women with diabetes treated only with tablets and those women without diabetes.

We went on to analyse how exposing placental tissue to these medications would affect the amount of transporters. We showed that not only did the individual medications leave the levels of transporter protein unaffected; they were also unaffected when the two medications were given in combination.

This work is important because it shows that giving these treatments during pregnancy is unlikely to put the unborn baby at unwanted risk from exposure to high levels of these tablets. We have also shown that combining the two tablets (an aspect of treatment that is currently being tested in pregnant women) does not decrease the amount of transporter proteins present in vitro and therefore is unlikely to increase the baby's exposure to one or both of the medications.

Acknowledgements

I would sincerely like to thank Professors Jane Norman and Rebecca Reynolds for supervising my work along the way; for their support and their great knowledge that they shared with me whenever it was needed.

I would also like to thank all the lab staff and post-doctorates working in the lab who had the patience to teach me how to perform the different experiments required for my research and how to go about solving problems when they arose. These people include Dr Forbes Howie, Dr Simon Riley, Dr Sara Rinaldi, Dr Marian Aldhous, Ms Rose Leask.

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Introduction

Gestational Diabetes Mellitus (GDM) affects between 3-10% of pregnancies with consequences for both the mother and the newborn infant [1]. The target of GDM management is achieving euglycaemia as this has been demonstrated to reduce adverse neonatal events including malformations, fetal macrosomia and hypoglycaemia [2].

With the increase in pressure on healthcare providers due to the increasing incidence of GDM, there is a drive to manage this condition in not only the safest way possible but also in a way which is acceptable to women, with the easiest route of administration of medication and dosing schedule to increase their concordance. Therefore, attention has turned to looking at the use of oral hypoglycaemic agents (OHAs) for the treatment of GDM. There are two classes of OHAs used in pregnancy; biguanides (for example metformin) and sulphonylureas (for example glibenclamide). Both oral agents have become recognised forms of treatment of GDM.

In the placenta there is a network of apical ABC transporters that are in place to protect the fetus from exposure to unwanted substances including medications such as metformin and glibenclamide. In this thesis, I have examined the mRNA and protein levels of these transporters in placentas from women with GDM, and the effect of in vitro treatment with metformin and glibenclamide on transporter expression to assess the impact of giving both these medications together in pregnancies complicated by GDM.

This report will begin with an introduction to GDM (chapter 1), followed by a review of the literature regarding the network of placental ABC transporters that transport glibenclamide and metformin across the placenta (chapter 2), characterisation of

transporter expression in placentae from diabetic and non-diabetic women (chapter 3) and also the effect of metformin and glibenclamide on transporter expression (chapter 4).

1. Gestational diabetes mellitus

Gestational Diabetes Mellitus (GDM) can be defined as carbohydrate intolerance of variable severity with onset or first recognition during pregnancy, presenting with elevated blood glucose [3]. In order to understand the management of GDM, it is first necessary to understand the pathophysiology behind the disease.

1.1 Pathophysiology

1.1.1 Action of Insulin

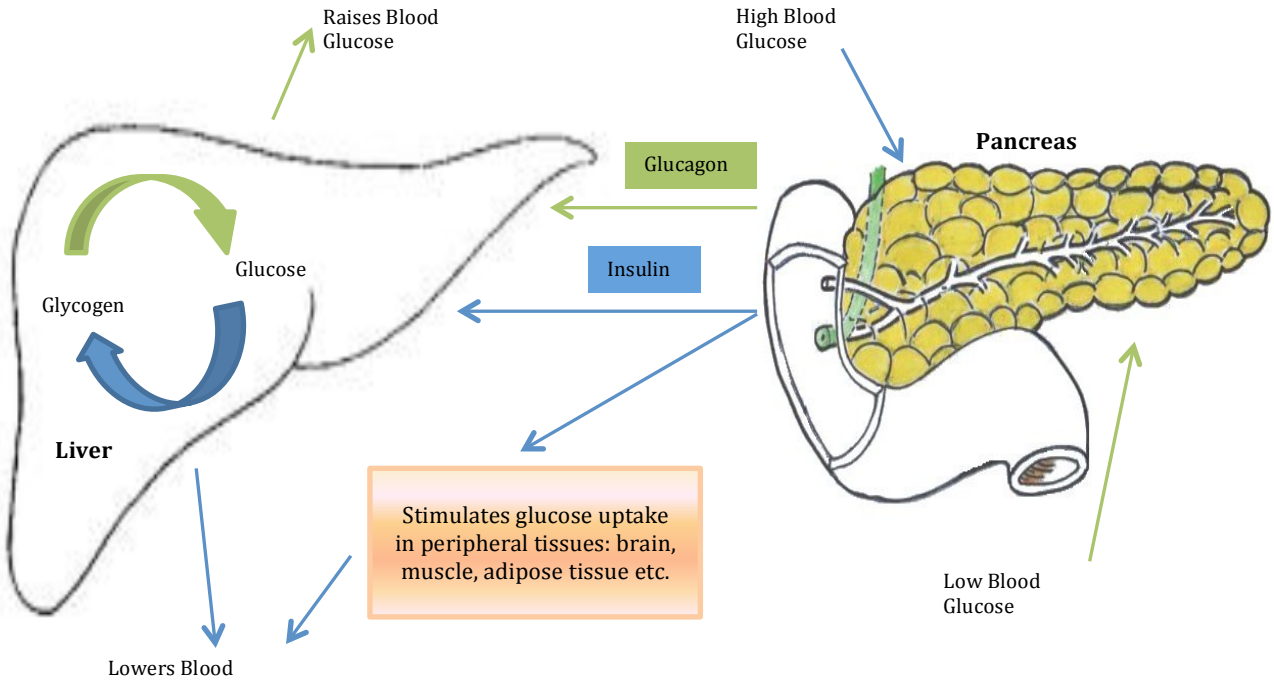


Figure 1: Schematic of action of insulin and glucagon and the effects on blood glucose levels in humans.

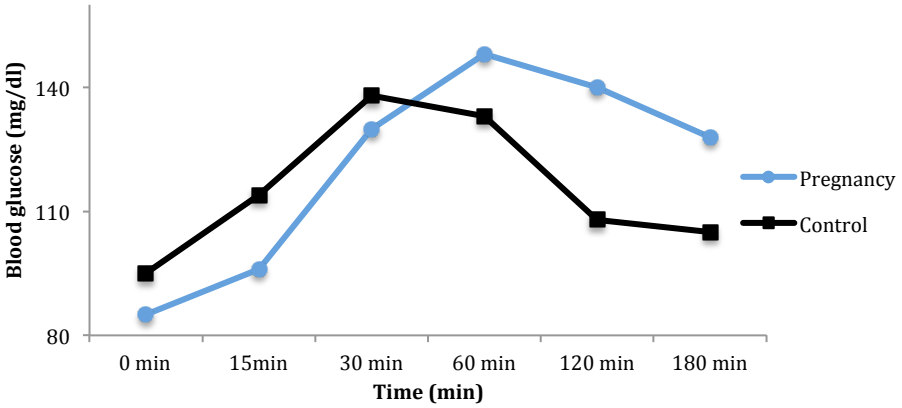
In people without diabetes the β-islet cells of the pancreas release insulin in response glucose and protein loads. With regard to glucose metabolism, insulin has two main functions (see blue arrows in Figure 1): stimulating peripheral glucose uptake, out of

the circulation and into cells; and inhibiting gluconeogenesis in the liver (glucose production) with the overall effect of decreasing blood glucose levels.

1.1.2 Normal Pregnancy

Pregnancy itself is a diabetogenic state, associated with increasing insulin resistance due to the insulin-antagonistic actions of the placental hormones human placental lactogen (HPL), progesterone and prolactin [4]. Throughout pregnancy, increasing levels of placental hormone production result in rising insulin resistance with gestation. In both non-diabetic pregnant women and women with GDM there is an almost doubling in fasting plasma insulin levels compared to the non-pregnant state [5]. It has been demonstrated that insulin production in response to glucose stimulus increases in pregnancy (as a result of β-cell hypertrophy and hyperplasia [6]), whilst the responsiveness of peripheral tissues and the liver to this higher insulin level is reduced [7]. Studies have shown that there is no change in insulin degradation in pregnancy [8].

A)



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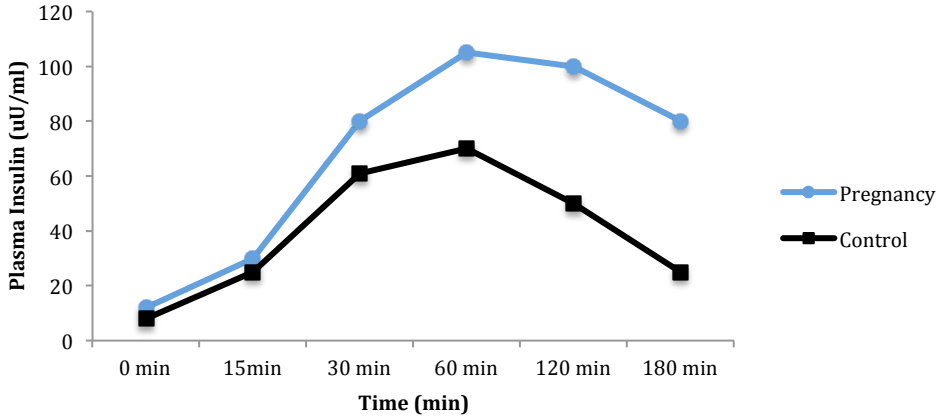


Figure 2: (A) Blood glucose and (B) plasma insulin concentrations after the oral administration of 100g glucose in control subjects and in 20- to 30-week pregnant women. (Adapted from Puavil et al.) [9]

Figure 2 demonstrates that whilst the insulin response to a glucose load may be higher in pregnancy, the blood glucose still increases more than in non pregnant subjects; implying a degree of insulin resistance. This is supported in figure 3 below where the changes in plasma insulin-glucose ratio (i.e. the degree of insulin resistance) during an oral glucose tolerance test (GTT) are noticeable even in early pregnancy but gradually deteriorate with increasing gestation.

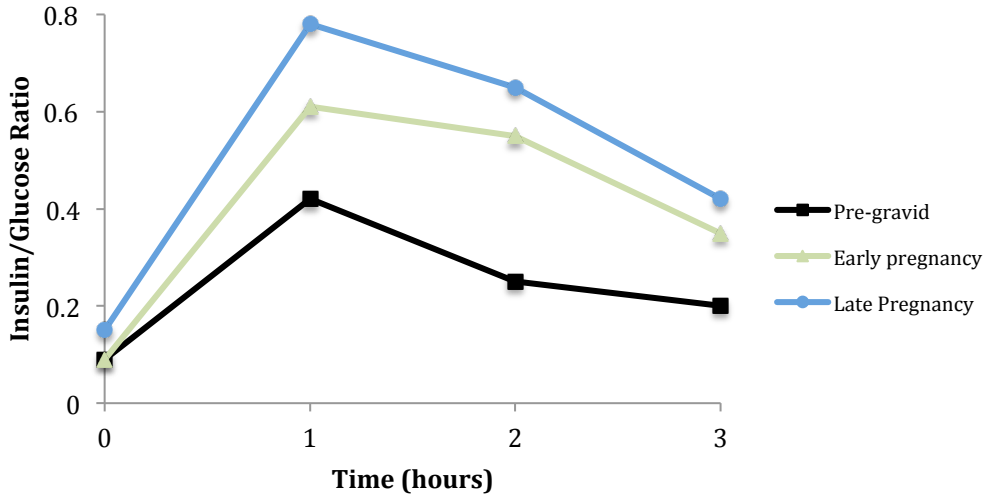


Figure 3: Longitudinal changes in plasma inulin-glucose ratio during OGTT (mean) in pre-gravid women, in early pregnancy (12-14 weeks) and late pregnancy (34-36 weeks). [Adapted from Catalano et al.] [10]

The hypothesis that the increased insulin levels seen are solely the result of higher background serum glucose levels in pregnancy has been disproven through demonstration that infusing glucose to achieve serum glucose levels that are comparable in women with GDM and normal glucose tolerant (NGT) pregnant women compared to those in the postpartum state results in significantly elevated insulin levels both in the GDM cohort (3 times increased) and NGT cohort (4 times increased) compared to the non-pregnant cohort [11].

1.1.3 Pregnancy with Gestational Diabetes

It has already been shown that pregnancy is a diabetogenic state; it is logical to follow-on that pathological processes can occur which result in the heterogeneous disorder which is GDM, either through a failure to make the necessary rise in insulin production demonstrated above, through a reduced peripheral responsiveness to endogenous insulin secretion (above and beyond that already seen in pregnancy) or through a combination of these factors, giving the spectrum of disease as is seen in GDM.

Non-obese women with GDM and pregnant women with NGT have comparable fasting insulin levels, approximately twice non pregnant (postpartum) levels [12]. However, obese women with GDM have significantly higher fasting insulin levels when compared to non-obese women with GDM and pregnant women with NGT[13].

Whilst women with GDM have significantly higher insulin response to a glucose load compared to postpartum, this insulin peak has been demonstrated to occur later than that in pregnant women with NGT [12]. Hence, a glucose load results in higher serum glucose for a longer period in women with GDM compared to those with NGT. Furthermore, whilst the absolute insulin response was comparable between pregnant women with GDM and pregnant women with NGT, the insulinogenic index (insulin response per unit of glycaemic stimulus) was significantly lower in GDM pregnancies (40%) compared to normal pregnancies (90%).

It has been demonstrated that during both early and more profoundly in late pregnancy, the first phase insulin response (insulin released immediately in response to elevated glucose levels as opposed to second phase response which is a slow continuous release independent upon glucose levels) is significantly reduced among women with GDM when compared to those with NGT[14].

Buchanan et al demonstrated a significant reduction in first-phase insulin response as well as a borderline-significant reduction in second-phase insulin secretion between GDM and control subjects, however no change in insulin sensitivity; concluding that GDM develops as a result of an impairment of β-islet cell function, rather than inappropriate insulin resistance [15].

As mentioned previously, insulin resistance increases in all pregnancies. It has been demonstrated that insulin sensitivity is equally reduced in normal pregnant women and women with GDM, as it demonstrated in table 1 below.

	Insulin Sensitivity ($min^{-1}.\mu U^{-1}.ml^{-1}.10^{-4}$)
Non-pregnant women	3.0 ± 0.6 (n=7)
Pregnant women with normal glucose tolerance	0.9 ± 0.3 (n=8)
GDM	1.1 0.2 (n=16)

Table 1: Insulin sensitivity among non-pregnant women, pregnant women with NGT and women with GDM. Mean ± SE (adapted from Buchanan et al [15]). There is no significant difference between the NGT pregnant women and the GDM group.

This fall in insulin sensitivity is compensated for by a marked increase in first and second-phase insulin response in women with normal glucose tolerance. However, as has already been demonstrated [15] some women are unable to mount this increased response, resulting in glucose intolerance and GDM.

It is only through an understanding of the pathogenesis of GDM that one can begin to contemplate the screening/diagnosis and management of the condition.

1.2 Consequences of GDM

Pregnancies in which the mother has GDM are at risk of a number of maternal complications including hypertensive disease and risk of caesarean section [16]. It has been hypothesised that fetal hyperinsulinaemia (to counteract elevated maternal and hence fetal circulating serum glucose levels) results in the following increased fetal risks [17]:

- Overgrowth of insulin-sensitive tissues such as adipose tissue, increasing the risk of fetal macrosomia, shoulder dystocia, perinatal death, birth trauma and need for caesarean section.
- Hypoxaemia in utero causing increased fetal death, fetal polycythaemia, hyperbilirubinaemia and renal vein thrombosis.
- Neonatal metabolic complications such as hypoglycaemia
- Increased long-term risks of obesity and type 2 diabetes in the child

It has been demonstrated that with interventions including diet and exercise modification, blood glucose monitoring and insulin treatment, with the intent of achieving euglycaemia, there is a significant reduction in the risk of serious perinatal outcomes (4% versus 1%, p=0.01) [2].

1.3 Treatment of GDM

The target of GDM management is achieving euglycaemia with a particular focus on reducing post-prandial episodes (at one and two hours) of hyperglycaemia as this is correlated better with certain adverse neonatal events including malformations, fetal macrosomia and neonatal hypoglycaemia [18].

	Blood glucose
Fasting	<5.5mmol/L
At <35 weeks gestation 2 hours post prandial	<7mmol/L
At >35 weeks gestation 2 hours post prandial	<8mmol/L
Or a post prandial value at any time	<9mmol/L

Table 2: Scottish Intercollegiate Guidelines Network (SIGN) recommendations for targets of glycaemic control in pregnancy [19].

Treatment of GDM currently begins with dietary modification and exercise advice. If this fails to achieve euglycaemia (approximately 50% of cases) then treatment with medications is indicated.

Traditionally the first-line medical treatment in the UK was with subcutaneous insulin injections [20]. Human (maternal) insulin does not cross the placenta due to its large molecular weight (5808Da) unless it is bound to IgG [21]. *In vivo* studies with insulin Lispro (Humalog), an insulin analogue, demonstrated no Lispro present in fetal cord blood [22]. By contrast, *in vitro* studies using a placental perfusion model, demonstrated the presence of insulin at 1-5% of maternal concentration in the fetal compartment. It should be noted that this was at concentrations exceeding those seen in the maternal circulation when insulin is administered therapeutically [21].

A part of the physiological process behind GDM is a reduction in the insulinogenic response to glucose stimulus; it is therefore logical that supplemental insulin, either short or long acting, will elevate circulating insulin levels. It is through these elevated levels that the reduction in insulin sensitivity seen in pregnancy (both in NGT women and women with GDM) can be overcome. Various studies have demonstrated the safety of insulin and insulin analogues in pregnancy and their safety has been well demonstrated in the treatment of pre-pregnancy (i.e. type 1 and 2) diabetes. There are

no adverse fetal outcomes associated with insulin therapy demonstrated in the literature.

Whilst insulin is well established in its ability to control GDM, there has been a drive to find treatments that are more acceptable to the patient, more cost effective and will also target the insulin resistance aspect of pregnancy that is exacerbated by the reduced insulin response seen in GDM. As a result of this attention has turned to the use of oral hypoglycaemic agents (OHAs) in pregnancy, with particular interest in metformin and glibenclamide (glyburide).

Metformin comes from the biguanide class of medications, increasing peripheral insulin sensitivity by activating AMP-kinase [23]. It stimulates glucose uptake by tissues, decreases hepatic gluconeogenesis and decreases intestinal glucose absorption [24-26].

Glibenclamide is a second-generation sulphonylurea which acts by inhibiting the ATP-potassium channel receptors in the β-islets cells of the pancreas to enhance endogenous insulin secretion [27].

In the last decade several trials have demonstrated the safety and efficacy for controlling hyperglycaemia with metformin and glibenclamide [28-33] in GDM.

The efficacy of glibenclamide (at a maximum dose of 20mg daily) was compared to insulin in a study involving 404 women with GDM; only 4% of women required additional insulin to achieve acceptable glycaemic control (as defined in table 2) and there was no difference in neonatal outcomes between the two treatment groups [28]. In contrast, several studies have since demonstrated a failure rate (the requirement of insulin therapy to achieve adequate glycaemic control) closer to 15-20% [34, 35].

The *Metformin versus Insulin for the treatment of Gestational diabetes* (MiG) trial was the first large randomised controlled trial (RCT) comparing the use of metformin with insulin in GDM [30] with the women receiving between 500 and 2500mg metformin daily. This trial used a composite of neonatal outcomes and they demonstrated no difference in adverse outcomes between the metformin group and insulin group (32.0% and 32.2% respectively, RR 0.99, 95% CI, 0.80 – 1.23). Among the women assigned to metformin, 46.3% required additional insulin therapy.

A comparative study between metformin and glibenclamide in 150 women with GDM demonstrated no difference between 1hr postprandial glucose values, rates of preeclampsia or maternal hypoglycaemia. 34.7% of patients receiving metformin required additional insulin therapy, in comparison to 16.2% in the glibenclamide group (p=0.01)[35]. The mean birth weight of babies from the metformin group was lower than that from the glibenclamide group (3103g ±600g versus 3329g ±334g, p=0.02) but otherwise there was no difference in neonatal outcomes. Whilst the requirement for additional insulin therapy in the metformin group was 2.1 times higher than the glibenclamide group, this did not have an impact upon clinical outcomes.

These studies have demonstrated not only the safety of these oral medications in terms of maternal and fetal outcome measures, but also the ability to achieve euglycaemia in line with insulin therapy. In these trials the range of metformin taken was between 500mg (one tablet) and 2500mg daily and the range of glibenclamide dosing was between 2.5mg (one tablet) and 20mg (four 5mg tablets) daily. In the MiG trial 1.9% of participants had to stop treatment due to gastrointestinal (GI) side effects and 8.8% had GI side effects that limited their dose escalation. 76% of participants stated they would chose metformin over insulin in a subsequent pregnancy. There is no mention in the glibenclamide versus insulin study [28] regarding patient tolerability of treatment

although the side effect profile is thought to be similar to that of metformin. In this study there was a lower rate of maternal hypoglycaemia in the glyburide group compared to the insulin group (2% vs 20% respectively, p=0.03).

2. Placental transfer of drugs and GDM treatment

2.1 Placental transfer of drugs

Metformin is a small, basic compound and as a substrate for organic cation transporters (OCTs) has been shown to cross the placenta [36] in a carrier-mediated fashion rather than by passive diffusion [37]. The level to which it passes and the concentrations that it can build to in fetal blood is controversial. In vivo data has demonstrated cord blood levels to be 60% that of maternal levels; taken from 23 babies whose mothers took metformin (median daily dose 2000mg) through their pregnancy [38]. However, this study only measured maternal metformin levels in seven of the women and the samples were taken at different times in relation to last metformin dose; cord blood on average 10 hours since last metformin dose and maternal blood 4.5hours after last dose. The half-life of metformin is between 1.5 and 4.5 hours, meaning this study may have under-estimated the level of fetal metformin exposure. Another study demonstrated levels higher in cord blood than maternal blood (up to 2 times increased) although this study is also limited by a small sample size (n=15) [39]. It has been hypothesised that fetal levels may exceed maternal levels due to fetal reabsorption of the metformin (from amniotic fluid) or due to maternal excretion [40].

In contrast to the in vivo data above, an in vitro study using placental dual perfusion studies demonstrated that metformin crosses into the fetal circulation, with a fetal to maternal concentration ratio of 0.51 ± 0.15 (mean ± SD) [40]. In keeping with other studies they demonstrated equal levels between umbilical artery and vein, suggesting minimal distribution and metabolism of metformin by the fetus. The difference between the in vivo and in vitro results may be as a result of a limited experimental

time, thereby excluding the effect described above of fetal reabsorption and maternal excretion.

There is scarce data as to whether increasing the insulin sensitivity of the fetus as a result of metformin exposure causes any harm. It has been hypothesised that there may be an adverse effect on fetuses in the situation of reduced placental perfusion and/or fetal growth restriction where an increase in peripheral insulin resistance is protective for fetal survival [41]. Vanky et al demonstrated that fetal exposure to metformin did not appear to alter umbilical blood pH (metformin has been known to induce lactic acidosis in adults) or cause teratogenicity, however recommended further longitudinal studies to look for long term effects of metformin on the fetus [39].

Initial studies looking at whether glibenclamide crossed into the fetal circulation suggested that any transfer was negligible; this was attributed to the thought that glibenclamide is predominately, 99.8%, protein bound and coupled with a short (six-hours) half-life makes for a very small amount of free drug available for placental transfer [28, 42, 43]. However, with the development of more specific methods for detecting glibenclamide at lower concentrations (for example high-performance liquid chromatography mass spectrometry assays with a limit of quantitation (LOQ) of 0.25ng/ml) it has emerged that it does in fact cross the placenta to some extent.

In a recent study of 40 pregnant women with GDM treated with glibenclamide, the presence of glibenclamide in cord blood was demonstrated, at levels on average 70% ± 40% that of the concentration in maternal serum [44]. Further studies have shown that glibenclamide crosses into the placental circulation via diffusion, however, much of it never reaches the fetal circulation as a result of a network of placental apical membrane transporters which actively efflux the drug back into the maternal circulation (against its concentration gradient) [45-48]. Additionally, glibenclamide

present in the fetal circulation has been shown to be transported against a concentration gradient into the maternal circulation [49]. These transporters are thought to play a key role in protecting the fetus from unwanted endogenous metabolites and xenobiotics (the presence of a chemical not normally expected to be found in an organism).

2.2 The Human Placenta

2.2.1 Placental Function

The human placenta is the organ by which the fetus is attached to the uterus (via the umbilical cord), bringing the fetal and maternal circulations into close contact. The placenta has many functions including supply of nutrition to the fetus, removal of metabolic waste products from the fetus, endocrine functions involving secretion of steroid and peptide hormones into maternal and fetal circulation and protection of the fetus against potentially toxic compounds.

2.2.2 Placental Structure

The placenta comprises of both maternal tissue (decidual tissue forming vascular units called cotyledons) and fetal tissues (forming the chorionic plate and chorionic villi). The functional unit of the placenta is the syncytiotrophoblast (ST) that is formed by the fusions of the cytotrophoblast (CT) stem cells forming an area devoid of lateral cell membranes, producing a single rate-limiting layer of multinucleated cells [50-52]. The ST is bordered by the brush border membrane (in direct contact with maternal blood) and the basal membrane (facing fetal circulation).

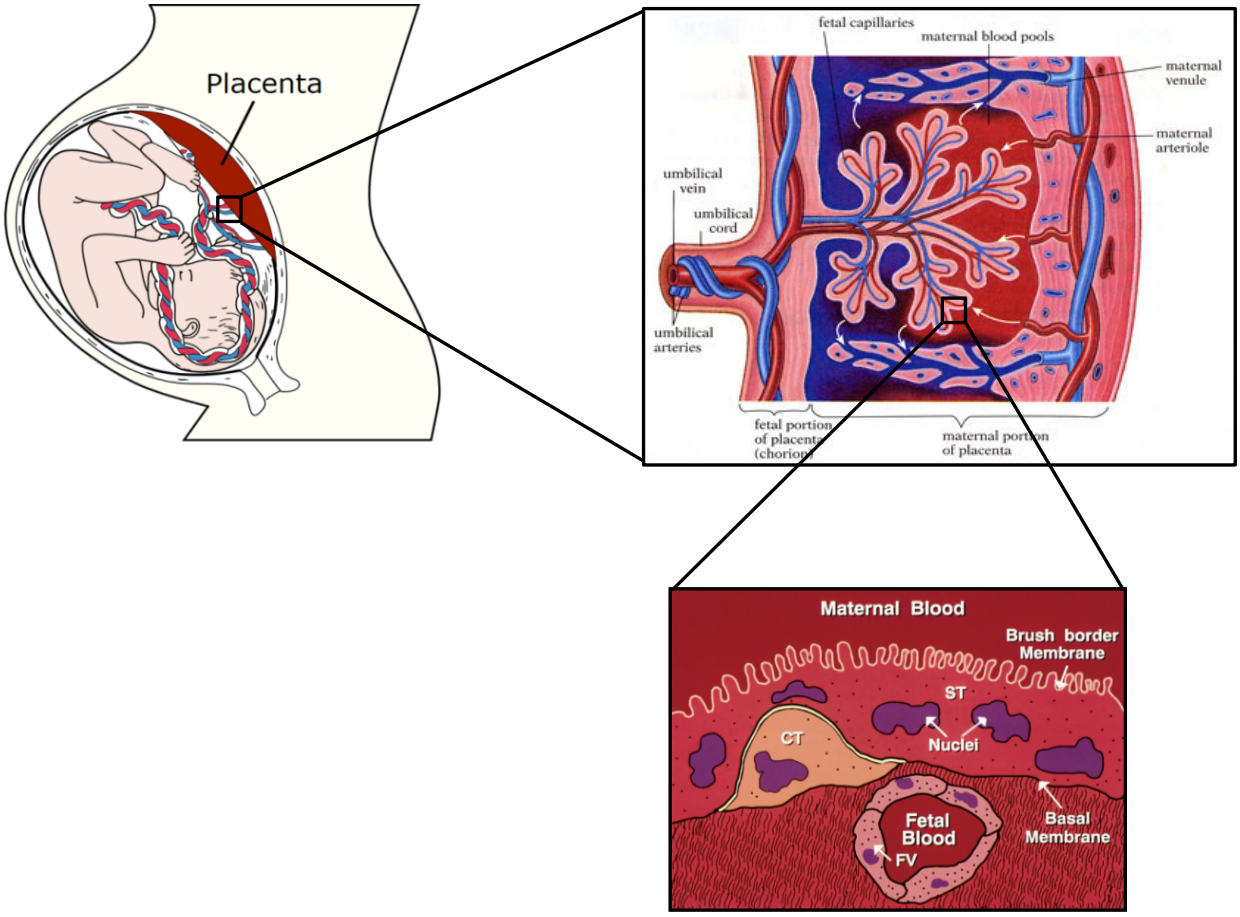


Figure 4 Placental structure demonstrating the different maternal and fetal components as well as the syncytiotrophoblast (ST) , cytotrophoblast (CT), fetal vein (FV) and the location of the two membranes which provide a barrier between maternal and fetal circulations; the brush border membrane and the basal membrane [53-55]

2.2.3 Placental transporters

It is recognised that a large variety of compounds in the maternal circulation can cross, to some extent, the placenta [50, 56]. This ability is dependent upon their lipid solubility, molecular size, plasma protein binding and degree of ionization [57].

Distributed across and between the brush border membrane and the basal membrane are a variety of enzymes, hormone receptors and transporters [58]. These transporters are involved in the transfer of nutrients to the fetus as well as the efflux of substances (endogenous or exogenous) from the fetoplacental unit into the maternal circulation.

It is often necessary to give medications to a woman during her pregnancy to treat a variety of conditions, such as GDM. It is the ability of many drugs to act as substrates to the placental transporters which allows for them to be effluxed away from the fetal circulation and thereby reducing fetal exposure to potentially harmful compounds [47].

The ATP-binding cassette (ABC) transporters have been identified on the placenta and various studies have demonstrated their involvement in the efflux of medications away from the fetus, including glibenclamide and metformin.

2.3 PLACENTAL ABC TRANSPORTERS

The ABC transporter super-family plays a crucial role in many important biological processes in both eukaryotes and prokaryotes [59] including a role in drug efflux. Though initially implicated in making cancer-cells resistant to anticancer drugs [48, 60] they have since been shown to have a role in many normal tissues of the body involved in the oral bioavailability, hepatobiliary, intestinal and renal excretion. They appear to be concentrated in organs involved in detoxification processes [61]. They also play an important role in protecting various sites such as the brain, testis and fetus from pharmacological influences [62]. It is this role of protecting the fetus from the effect of xenobiotics [46, 47] that this review will be focusing on, with particular interest in three of the ABC transporters as they are implicated in the efflux of metformin and glibenclamide:

- P-glycoprotein (P-gp); *ABCB1/MDR1* gene product
- Multidrug resistance-associated protein 1-3 (MRP1-3); *ABCC* gene product
- Breast cancer resistance protein (BCRP); *ABCG2* gene product

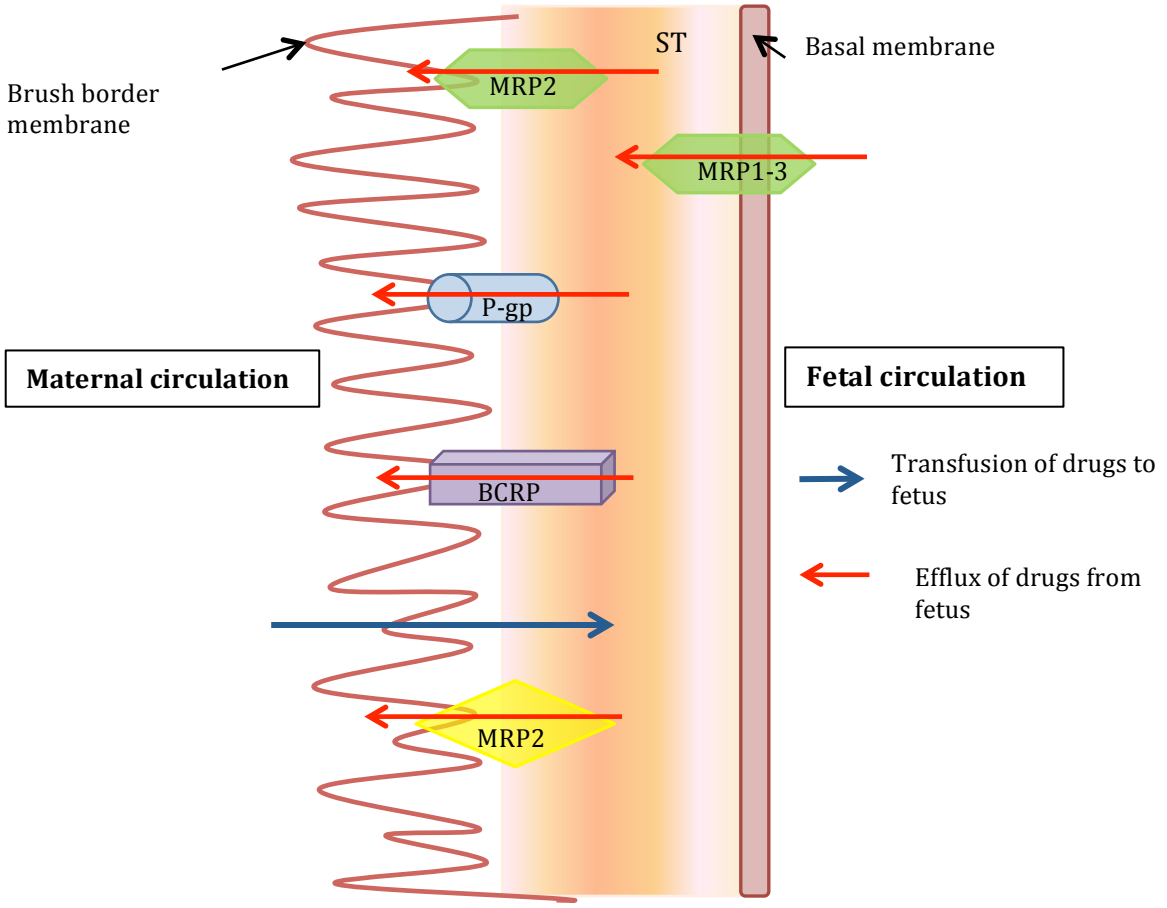


Figure 5: Schematic representation of location of main ABC transporters of interest in this report

2.3.1 P-glycoprotein (P-gp)

P-gp is a 170kDa membrane protein first discovered in 1976 in Chinese hamster ovarian tissue [63]. P-gp acts to extrude a large number of substances from the syncytiotrophoblast, typically favouring hydrophilic positively charged compounds [64].

Evidence of placental P-gp presence

The presence of P-gp protein in the placental trophoblast was first demonstrated through immunohistochemistry in 1988 and again in 1990 [61, 65]. Since then numerous studies have confirmed its presence, both at mRNA and protein levels [60,

66-68]. Whilst carrying out functional testing of placental P-gp in placental brush border membrane vesicles and basal membrane vesicles, Ushigome *et al* demonstrated that P-gp is located on the maternal surface of the trophoblasts (brush border membrane) and not the basal membrane [69]. This was confirmed by Atkinson *et al* [67], suggesting that the maternal brush border membrane plays an active role in preventing the accumulation of xenobiotics in the syncytiotrophoblast and hence, protecting the fetus.

P-gp expression through gestation

P-gp expression falls significantly during the pregnancy; using western blot analysis a 2 fold decrease in P-gp expression (at protein level) ($p=0.0004$) between first and third trimester placentas was demonstrated [70]. This was confirmed through the use of real-time PCR and western blot analysis, demonstrating a significant difference in placentas from the first and second trimesters compared to term at both protein and mRNA level [71]. A further study also confirmed these findings, using real-time PCR, western blot analysis and ELIZA, showing P-gp levels 44.8-fold higher at 60-90 days gestation compared to term placentas ($p<0.05$) [72].

This reduction in P-gp levels through gestation be a representation of the fetus' greatest susceptibility to the teratogenic effects of xenobiotics in the first trimester but could produce concern regarding the possible transfer of xenobiotics (from drugs given to the mother) to the fetus nearer term.

P-gp activity

In vivo studies

Lankas *et al* first demonstrated the protective properties that P-gp provides to the fetus in mouse studies. Using CF-1 mouse stock (25 % P-gp deficient, phenotypically similar

mdr1a -/- knockout mice, 25% *mdr1a* +/+ and 50% heterozygous *mdr1a* +/-) they examined the impact of antenatal exposure to avermectin (known to cause cleft palate malformation in fetus). They found that fetuses that had a P-gp genotype of -/-, 100% had cleft palate whilst those with +/+ were completely protected. Within individual litters, fetal exposure and levels of avermectin were dramatically different between the different fetal P-gp genotypes (+/+ vs. +/- vs. -/-, $P<0.05$) concluding that the degree of fetal exposure was related to the level of fetal *mdr1a* P-gp expression [47].

A recent review addressed the use of mouse models for human placental transport studies [73]. Both human and mouse placentas are haemochorial type (i.e. fetal trophoblast tissue is bathed in maternal blood) [74] and whilst there may be structural differences, it would appear that there are also significant similarities. Whilst there are two additional layers of trophoblast in the mouse placenta compared to the human placenta, it has been demonstrated through the use of electron microscopy and alkaline phosphatase staining, that the maternal facing trophoblast layer in the mouse is equivalent to the microvillus (maternal facing) layer in the human syncytiotrophoblast [75]. Various studies have been carried out looking at nutrient transport across human and mouse placentas, demonstrating that the two additional trophoblastic layers do not have a large impact upon permeability and thus the utility of the mouse-model for transport studies [76]. Whilst mouse-models can provide a useful means for performing in vivo studies to look at the activity of ABC transporters in the placenta, the results cannot necessarily be extrapolated directly to human studies.

In vitro studies

In vitro dual placental perfusion (DPPL) models (using rat placentae) were used to demonstrate P-gp action to export drugs into the maternal compartment [77]. The use of ATP inhibitors (sodium azide) demonstrated that the transfer of the P-gp substrate

into the fetal compartment was significantly increased (more than twice, $P<0.01$) compared to controls highlighting the fact that the P-gp transporter is ATP-dependent. Through the use of P-gp inhibitors it was demonstrated that the amount of substrate entering the fetal compartment was significantly higher (1.7-1.8 times, $p<0.01$) when the P-gp inhibitor was present in the maternal compartment compared with controls. This was increased further when there was presence of the inhibitor in both maternal and fetal compartments, leading to a three-fold increase in transplacental transport of P-gp substrate into the fetus ($p<0.01$). This was confirmed in a study using placental vesicles and hydatidiform mole tissue; the presence of P-gp in placental tissue and the transport of [^{3}H]vincristine across the membrane, in the in the presence of ATP, by these transporters [68].

The activity of P-gp in the placenta has also been demonstrated using BeWo cell lines (b30 clone), originally obtained from human choriocarcinoma and known to be a good model for in-vitro studies of placental transport [78]. Using the BeWo cell line transfusion studies demonstrated that in the presence of cyclosporin (a P-gp inhibitor), the transport of three different P-gp substrates was significantly reduced from a fetal to maternal direction, whereas there was no impact on transport from a maternal to fetal direction [69].

2.3.2 Breast cancer resistance protein (BCRP)

BCRP is the most recently discovered member of the ABC family of transporters, first cloned in 1998 [79]. BCRP shares some overlapping substrates with P-gp and also has some that are distinct [79-81].

Evidence of placental BCRP expression

BCRP has been shown to be expressed in high levels in placental tissue both using mRNA studies [79, 82] and immunohistochemistry. BCRP is predominately located in the maternal brush border membrane facing maternal blood [83]; unlike P-gp there is also a small level of BCRP present on fetal capillary endothelial cells [84].

BCRP expression through gestation

There is a discrepancy in the evidence regarding the expression of BCRP through gestational age. A quantitative study into the expression (protein and mRNA) of BCRP demonstrated through both ELISA and Western Blot Analysis that placental expression of BCRP is variable but independent of gestational age [72]. This was confirmed in a further study using rtPCR to look at the mRNA levels of BCRP that found levels did not change significantly as gestation progressed; however, did demonstrate with the use of Western Blot analysis that the protein levels did increase towards term [84]. Contrary to these studies, a further study demonstrated a trend similar to that with P-gp: the BCRP mRNA levels in human preterm placentas (28 ± 1 weeks, 15 placentas) were approximately two times higher than that at term (39 ± 2 weeks, 29 placentas), $p<0.05$ [85]. The reasons behind the variation in the results between the studies is unclear, although the first two studies both had much smaller sample sizes than the latter, although that study only looked at per-term and term placentas and not placentas from early pregnancy. The data from rodent studies is consistent with that found in the latter study, demonstrating a peak in BCRP expression mid-trimester and reducing again towards term [86].

BCRP placental activity

In vivo studies

Jonker *et al* carried out the first *in vivo* transport study demonstrating the activity of BCRP in the placenta [87]. They used P-gp deficient mice (by excluding this transporter, they were ensuring that the BCRP substrate used would need to be transported by BCRP alone) and exposed them to GG918 (a BCRP inhibitor). They then measured the distribution of topotecan (a BCRP substrate) in the fetuses and found a two-fold increase compared to vehicle-treated mice ($P<0.01$).

This work was confirmed in a study looking at the accumulation of glibenclamide in Bcrp1 -/- pregnant mice and wild-type mice. They demonstrated that the concentration ratio in Bcrp1 -/- mice was approximately 2.5 times higher than that of wild-type pregnant mice ($p<0.05$) [88].

In vitro studies

Staud *et al* demonstrated through the use of dually perfused rat placentas and rat HRP-1 placental cell lines (express BCRP but not P-gp) that BCRP effluxed substrates from the fetal to the maternal circulation, even against a concentration gradient (fetal to maternal clearance of cimetidine, a BCRP substrate, was 25 times higher than maternal to fetal transfer) [89].

HEK cell lines (known to have BCRP expression with little endogenous P-gp, MRP1 or MRP2 expression) were used to show that inhibition of BCRP transporters increased the intracellular accumulation of MX fluorescence two-fold ($p<0.01$) [88]. This study also demonstrated through Transwell transport studies that the transport of glibenclamide in a fetal to maternal direction was significantly reduced in the presence

of a BCRP inhibitor, while there was no difference in maternal to fetal movement of glibenclamide ($p<0.005$).

Gedeon *et al* used in-side out membrane vesicles from human placental tissue to explore the transport of glibenclamide across several ABC transporters [90]. They demonstrated that in the presence of novobiocin (a BCRP inhibitor) there was a three-fold increase in intracellular accumulation of glibenclamide ($p<0.01$).

2.3.3 Multidrug Resistance Proteins (MRP1, 2 and 3)

The MRP family was discovered in 1992 initially by Cole *et al* who identified MRP-1 [91]. Gradually over the ensuing six years the other members of the family (MRP 1-7) were identified [92-96]. The corresponding sequences for these members were identified by Allikmets *et al* in 1996 [97]. MRP proteins mediate ATP-dependent transport of compounds that are unconjugated, amphiphilic anions of lipophilic compounds conjugated to glutathione, glucuronate and sulphate [98].

Evidence of MRP1-3 expression in the placenta

The localisation of MRP transporters is not quite as clear-cut as that of BCRP and P-gp. *In vitro* studies using BeWo cell lines have confirmed the presence of MRP1 mRNA in placental tissue [93, 99]. St-Pierre *et al* used immunohistochemistry to demonstrate the presence of MRP1, 2 and 3 in the placenta [48].

St-Pierre *et al* localised MRP1 to the fetal endothelial cells, a finding consistent with Nagashige *et al* who used Western blotting analysis and immunohistochemistry to demonstrate this. They were also able to localise MRP1 further to the luminal side of the fetal blood endothelial cells in the villi [100]. Where St-Pierre and Nagashige differed is in the localisation of MRP1 in the trophoblast; whilst St-Pierre *et al* demonstrated the apical (brush border membrane) expression of MRP1, Nagashige *et*

al localised MRP1 to the basolateral membrane (fetal side). This finding would be more in keeping with other tissues where MRP1 is located on the basolateral side of tissues [101]. A further study using western blotting and immunohistochemistry staining showed the optical density (on western blotting with densitometry) of MRP1 on the basolateral membrane to be 8.8 ± 1.4 compared to 2.3 ± 0.3 ($p<0.005$) on the brush border membranes [67].

MRP expression through gestation

Pascolo et al performed PCR studies looking at MRP expression in placental tissue at different gestations [99]. MRP 1 expression has been shown to increase through gestation, with levels four-fold greater in the third trimester compared to the first trimester ($p<0.0001$). Levels of MRP2 also increased two-fold with gestation, (4.9 ± 0.6 (term) vs. 2.65 ± 0.3 (early gestation), $p < 0:004$). By comparison, there was no demonstrable difference in MRP3 expression in early gestation and term placental tissue.

MRP placental activity

BeWo and Jar choriocarcinoma cell lines (shown to express MRP 1 and minimal P-gp) were used to demonstrate that [^{3}H] vinblastine (a MRP 1 substrate) transport was inhibited in the presence of cyclosporine A (a MRP 1 inhibitor) leading to an intracellular accumulation of [^{3}H] vinblastine ($p<0.0001$).[67].

A study looking at the transport of three OHAs (rosiglitazone, glibenclamide and metformin) demonstrated the transport of glibenclamide via MRP1 transporters in the presence of inhibitors of P-gp and BCRP [102]. The results from this study should be taken with some caution for the following reasons: The study used placental brush border, which it has already been demonstrated has a low prevalence of MRP1 and no

tests were carried out to check the presence of the transporters in the tissue prior to do the functional testing; they also utilised indomethacin as a MRP1 inhibitor although this is an inhibitor of MRP 2 and 3 as well.

St-Pierre et al also performed transport studies showing the uptake of two MRP substrates ([^{3}H]estradiol–17-β and DNP-SG). They performed the uptake studies in the presence and absence of ATP, demonstrating the ATP-dependence of these transporters. They did acknowledge that [^{3}H]estradiol–17-β was also a weak substrate for P-gp which was present in their samples therefore the rate of transport could not be attributed solely to MRP.

Gedeon *et al* demonstrated the transport of glibenclamide in MRP3 overexpressing cell lines [90]; in MRP3 transfected cells there was a 1.8-fold increase in accumulation of glibenclamide in the presence of indomethacin (an MRP inhibitor) ($p<0.05$) whilst in MRP1 transfected cells the opposite was true, with reduced accumulation of glibenclamide in the presence of indomethacin. This has also been demonstrated for MRP 3 and MRP 1 and 2 through other studies [99, 103]. However, in 2008 Gedeon *et al* used a dually perfused placental model to study the efflux of glibenclamide in the presence of Indomethacin (an MRP inhibitor) [104]. Interestingly this work showed no change in the transport of glibenclamide in the presence of indomethacin in tissues with biologically relevant levels of the transporters (unlike studies using over-expressing cell lines). This suggests that the previous *in vitro* studies were perhaps overestimating the role of the MRP transporters in the placenta, a finding exacerbated by the use of drug levels exceeding those that would be found clinically.

	P-gp	BCRP	MRP
Presence in placenta	Located on brush border membrane (maternal surface)		MRP 1 – located mainly on fetal endothelial cells MRP 2 – brush border location. MRP 3 – brush border and fetal endothelial
Gestational variation	Decreases through gestation	Peak mid trimester then decreases to term	MRP 1&2 – increases with gestation MRP 3 – no change with gestation
Activity	Actively (ATP dependent) extrudes substances from fetal to maternal circulation, *in vivo* and *in vitro* evidence.		Cell lines demonstrated activity however to lesser extent than P-gp. ATP dependent activity.

Table 3: Summary of ABC transporters of interest main characteristics discussed in above chapter

2.4 Placental ABC transporters and the transport of Glibenclamide and Metformin

Metformin has been shown to be actively transported across the placenta via transport carriers in an asymmetric manner with higher transport rates in the fetal to maternal direction than vice versa. An in vitro study using placental brush border inside-out vesicles examined the importance of the three main ABC transporters on the efflux of metformin [102]. Through the use of inhibitors of P-gp, BCRP and MRP they demonstrated the role these different transporters play in the efflux of metformin, showing that P-gp transports the majority of metformin, with BCRP also transporting some, when all three transporters were inhibited, the transport of metformin, to the maternal side, was reduced by 89±6%. The relative contributions of the different transporters can be seen in figure 6 below.

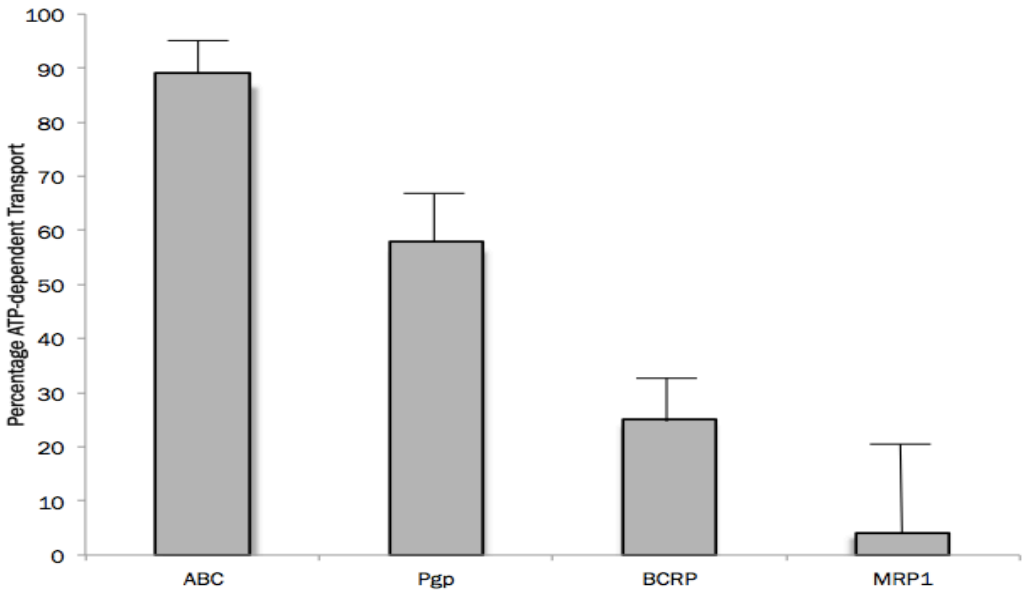


Figure 6: The relative contributions of P-gp, BCRP and MRP1 to metformin efflux from the fetal to maternal compartment, determined using chemical inhibition of ATP-dependent transport of 100nM [14C]-metformin in placental inside out vesicles (IOVs) (pool of 60 preparations). *Adapted from Hemauer S.J. et al 2010 [102]*

Compared to metformin there have been numerous studies looking at the efflux of glibenclamide across the placenta. Glibenclamide is highly lipophilic and like metformin has a low molecular weight. Kraemer *et al* provided the first evidence, using a dual perfusion of placental lobule (DPPL) technique with glibenclamide in both maternal and fetal circulations, that glibenclamide is actively extruded from the fetal circulation into the maternal circulation even against a concentration gradient [45]. They also carried out a parallel study in the presence of Verapamil, a known P-gp inhibitor, and demonstrated that this did not affect glibenclamide transport, suggesting another placental transporter transports the glibenclamide.

In 2006 Gedeon *et al* demonstrated through an in vitro study, using over-expressing cell lines, that inhibition of BCRP with novobiocin, leads to a pronounced intracellular accumulation of glibenclamide (3-fold increase) [90]. They confirmed the effect of P-gp inhibition on glibenclamide transport (i.e. it did not effect the transport) that Kraemer *et al* had demonstrated. Interestingly, in keeping with the findings of Payen *et al* [105] and Golstein *et al* [106] they showed that glibenclamide was an inhibitor of MRP 1, 2

and 3 as well as the other ABC transporters. It is important to note thought that the concentration of glibenclamide to bring about this effect was significantly higher than any therapeutic level (in the experiment they used a concentration of 98,800ug/L (200uM) whilst clinically the upper limit of glibenclamide measured in serum is 150ug/L).

Zhou *et al* performed the first in vivo studies, using a mouse model, to demonstrate the efflux of glibenclamide by BCRP, with fetal levels of glibenclamide two-fold higher in BCRP1-/- mice compared to wild-type ($p<0.01$) [88]. Again they confirmed the finding of previous studies that at doses far above those achieved therapeutically, glibenclamide is an inhibitor of the ABC transporters. Pollex *et al* provided the first *in vitro* evidence using DPPL that BCRP actively effluxes glibenclamide [107]. Interestingly, the use of nicardipine, a BCRP inhibitor, was able to partially block the transfer of glibenclamide across the placenta but not entirely, suggesting the presence of another transport mechanism or perhaps reflecting inter-individual transporter variability.

2.5 Placental ABC transporters and gestational diabetes

Nanovskaya *et al* compared transplacental glibenclamide transport between placentas from uncomplicated pregnancies and pregnancies complicated by GDM, demonstrating no difference between the groups [49]. Anger et al examined the expression of ABC transporters in placentas from women with GDM, Type 1 diabetes (both groups insulin treated) and those without diabetes (35 placentas included in study) using qPCR and Western Blotting [108]. They demonstrated that at the mRNA level, there were significantly increased levels of MDR1 (P-gp) in the GDM (on insulin) group (n=13) compared with the Type 1 DM (n=8) group ($P<0.01$) and control groups (n=14)

($p<0.05$). Interestingly though this did not translate to protein expression with no difference detected between the groups for any of the ABC transporters examined (BCRP, P-gp and MRP2)($p>0.05$). However; within the diabetic groups there was a positive correlation between maternal HbA1c level (a measure of glycaemic control) and BCRP mRNA and protein expression suggesting that levels change with glucose control($r=0.58$, $p<0.01$). A criticism of this study is that no effort was made to match the samples for gestation or BMI and there was a significant difference between the BMI of the GDM group and that of the control group (controls: 23.2 ± 4.5, GDM: 29 ± 6.6, $p<0.05$).

2.6 Conclusions

GDM is increasing in prevalence and the drive to develop treatments that are acceptable to the women, more cost effective and achieve euglycaemia whilst not compromising fetal safety is increasing. Clinical trials have been carried out demonstrating the efficacy of metformin and glibenclamide in the treatment of GDM compared to insulin and also demonstrated no adverse fetal outcomes with these treatments. These have both now become commonly used treatments.

The presence of ABC transporters in the placenta is now well documented although there are discrepancies in the evidence, particularly regarding the location of the individual transporters and their expression through gestation. There is some in-vitro evidence regarding the efflux of metformin from the fetal to maternal circulation via the P-gp transporters. There is far more evidence regarding the transport of Glibenclamide, with the body of evidence suggesting that it is predominately transported via BCRP. It has been postulated that both metformin and glibenclamide act as substrates for more than one ABC transporter.

The study by Anger et al only used placentas from women with diabetes treated with insulin and there is no evidence as to whether levels of these transporters also differ in women on diet control or OHAs. Further examination of the impact of glycaemic control and transporter expression would be beneficial and could translate clinically into identifying those women at increased risk of fetal exposure to xenobiotics if they have poor glycaemic control.

Whilst the transport of metformin and glibenclamide have been studied individually, it would be interesting to examine the impact of combined therapy on the ABC transporters involved in effluxing them from the fetal circulation as this is a treatment used in type 2 diabetes and an area of treatment for GDM which is currently undergoing clinical trials.

3. Investigation of ABC transporters in placentas from women with GDM and those without diabetes

As mentioned above, there is scanty evidence regarding the effect of gestational diabetes on the ABC transporters with the study by Anger et al showing some impact among a small cohort of placentas in insulin-treated patients. Despite a lot of research into the safety of oral hypoglycaemic agents (OHA's) for the treatment of GDM, and work demonstrating the importance of the ABC transporters for effluxing these medications away from the fetal circulation, there is a distinct lack of evidence regarding the impact of GDM and the OHA's used to treat it on the transporters themselves.

As a result the following experiments were designed and conducted, to examine the impact of GDM on placental ABC transporter expression:

1. Rt qPCR to quantify mRNA levels of *ABC B1* (P-gp), *ABC G2* (BCRP) *and ABC C3* (MRP3) in placentas from women with GDM and those without diabetes.
2. Western blot analysis to examine whether any differences in mRNA expression are translated into differences at a protein level.

3.1 Hypothesis

Expression of ABC B1 is elevated in placentas from women with GDM compared to non-diabetic women at the mRNA level but not at a protein level, however; the levels of ABC G2 and ABC C3 transporters are not altered.

This hypothesis is based upon the results from the Anger et al study [108] that had these results looking at placentas from women with insulin-treated diabetes when compared to placentas from non-diabetic women.

3.2 METHODS

3.2.1 Sample Acquisition

72 placental samples were obtained from the Edinburgh Reproductive Tissue Biobank (ERTBB), following unit policy and in accordance with West of Scotland REC 4 ethics approval. Immediately following delivery 4 full thickness cores were obtained from the four quadrants of the placenta, avoiding the chorionic plate and preserved in RNAlater. They were then snap frozen and stored in -80°C. Samples from women with GDM and non-diabetic control patients were matched for gestation at delivery and BMI with 36 samples in each group[1].

3.2.2 RT qPCR method

RNA extraction

Approximately 30mg of placental tissue was homogenised in 600uL of buffer RLT+βME mix (10uL β-mercaptoethanol to each 1ml buffer RLT) in a 2.0ml Eppendorf tube using the Qiagen Tissue Lyser at 25Hz for two minutes. The lysate was then centrifuged for 3 minutes at full speed and the supernatant removed and placed in a new 2.0ml Eppendorf tube. Total RNA was then isolated using the Qiagen RNeasy mini-kits (Qiagen, Australia) using the following method. The RNA was precipitated out of the lysate into a RNeasy spin column using 600uL 70% ethanol and centrifuged for 1min at ≥10,000rpm, flow-through was discarded. The spin column was then washed with

[1] This was using all the GDM samples that were held in the ERTBB at the time of commencing the study. No power calculation was performed.
[2] This dilution was found to be necessary as the protein solutions otherwise had a higher

350uL buffer RW1 (centrifuged for 1 minute at ≥10,000rpm again) to remove any material not well bound to the spin column, prior to a DNase step being performed to remove any genomic DNA present. 70uL buffer RDD was added to 10uL DNase 1 stock solution (gentle pipette mixing the samples, avoiding vigorous shaking as this can denature the enzyme) and then added to the spin column membrane and left for 30 minutes at room temperature. Further washes were then performed, first with 350uL RW1 buffer, centrifuged for 1 minute at ≥10,000rpm (flow-through discarded) and then with 500uL RPE buffer and centrifuged for 1 minute at ≥10,000rpm (flow-through discarded). This RPE buffer step was then repeated and centrifuged for 2 minutes at ≥10,000rpm; the RPE buffer contains ethanol therefore acts to wash and precipitate RNA to release it from the column but not wash it through (i.e. loosens it). The spin column was then removed and placed in a new 2ml collection tube and centrifuged for 1 minute at ≥10,000rpm to ensure all wash solutions were removed. The column was then placed in a 1.5ml Eppendorf tube and RNA extraction performed by adding 50uL RNase-free water directly to the spin column and centrifuged for 1 minute at ≥10,000rpm, the flow-through was retained as this now contained the extracted RNA which has been eluted from the column by the RNase-free water. This step was then repeated to ensure all RNA was removed. A Nanodrop 1000 was then used to check the quality of the RNA and calculate the concentration of total RNA (ng/uL), using 2uL samples of the RNA extracted, ensuring that the Nanodrop was calibrated first using 2uL of RNase-free water. Samples were then placed in a -80˚C freezer until needed.

cDNA synthesis

Prior to commencing the bench was wiped with RNase Zap and cDNA synthesis was performed on ice using high capacity reverse transcription kit (Applied Biosystems).

The cDNA synthesis kit and RNA samples were thawed on ice and then centrifuged at ≥10,000rpm for 5 seconds . 2X Mastermix was prepared in 1.5ml Eppendorf tubes using the quantities outlined in the table below, making enough for the number of RNA samples and two extra. Two samples worth of reverse transcriptase negative mastermix were made to act as a control. Once the mix was made, each Eppendorf tube was vortexed (on a vibrating plate) for 5-10 seconds to mix and then centrifuge at ≥10,000rpm for 5 seconds.

	For 20uL samples		**For 50uL samples**	
	RT pos (uL)	**RT neg (uL)**	**RT pos (uL)**	**RT neg (uL)**
10 x RT buffer	2	2	5	5
25 x dNTPs	0.8	0.8	2	2
10 x random primers	2	2	5	5
Reverse transcriptase	1	nil	2.5	Nil
RNase inhibitor	1	1	2.5	2.5
DEPC water	3.2	4.2	8	10.5

Table 4: Quantities of different components for making cDNA mastermix.

The amount of RNA (uL) needed to get a concentration of 1ug/uL was calculated (=1000/concentration RNA in sample from nanodrop(ng/uL)). This amount of RNA from each sample was added to labelled 0.2ml Eppendorf tubes; for 20uL reactions enough RNase-free water was added to make up to 10uL, for 50uL reaction enough RNase-free water was added to make up to 25uL. Mastermix was added to each tube; if 10uL mastermix for the 20uL reactions and 25ul mastermix for the 50uL reaction. NB at this point RT negative mastermix was added to the correct tubes labelled RT negative to create controls. The tubes were vortexed for 5 seconds and then centrifuged for 10 seconds at ≥10,000rpm. All samples were then placed for incubation in the G-storm thermal cycler on following settings: 25˚C 10mins, 37˚C 120mins, 85˚C 5mins. The cDNA was then used or stored at -20˚C.

RT qPCR

Prior to performing PCR I decided firstly to use pre-validated gene assays, thereby not needing to perform standard curves. 3 different housekeeping genes were assessed for endogenous controls, looking for consistency between diabetic and non-diabetic samples: *18S, YWHAZ* and *β-ACTIN*. *YWHAZ* was chosen for use due to its consistency between diabetic and non-diabetic samples and also it diluted out appropriately at a 1in10 dilution. To standardise across all the PCR plates, I ran the same non-diabetic control sample on each plate.

Pre-designed, validated gene-specific TaqMan® MGB FAM labelled primer and probe sets were used for *YWHAZ* (Hs03044281_g1), *ABC B1* (Hs00184500_m1), *ABC C3* (Hs00978473_m1) and *ABC G2* (Hs01053790_m1). The components for mastermix and cDNA samples were thawed on ice. A mastermix was made for each primer/probe in a 2.0ml Eppendorf tube, using 10uL of TAQman universal mastermix II (Applied Biosystems) with 1uL of primer/probe solution and 8uL of nuclease-free water (enough multiples of this was made for each primer/probe for each sample being tested). This was then vortexed for 5-10 seconds prior to being centrifuged for 10 seconds at ≥10,000rpm. Light exposure to the primer/probe mastermixes was kept to a minimum using silver foil and samples were kept on ice prior to being used in the plate. 1uL of cDNA was added to the relevant wells in a 384 well plate with each sample of cDNA being tested in triplicate. 19uL of mastermix was then added to each well for the relevant gene. An example layout of a PCR plate template is given below. If the plate was not being used immediately it was placed in the fridge at 5˚C for up to 72 hours, covered in silver foil.

N3 acting as calibrator across all plates

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	N3 P-gP			N3 BCRP			N3 MRP			N3 YWHAZ			N3 ACTIN			D9 P-gp			D9 BCRP			D9 MRP		
B	D1 P-gP			D1 BCRP			D1 MRP			D1 YWHAZ			D1 ACTIN			D9 YWHAZ			D9 ACTIN					
C	N1 P-gP			N1 BCRP			N1 MRP			N1 YWHAZ			N1 ACTIN			N9 P-gp			N9 BCRP			N9 MRP		
D	D4 P-gP			D4 BCRP			D4 MRP			D4 YWHAZ			D4 ACTIN			N9 YWHAZ			N9 ACTIN					
E	N4 P-gP			N4 BCRP			N4 MRP			N4 YWHAZ			N4 ACTIN			D11P-gp			D11 BCRP			D11 MRP		
F	D5 P-gP			D5 BCRP			D5 MRP			D5 YWHAZ			D5 ACTIN			D11 YWHAZ			D11 ACTIN					
G	N5 P-gP			N5 BCRP			N5 MRP			N5 YWHAZ			N5 ACTIN			N11 P-gp			N11 BCRP			N11 MRP		
H	D6 P-gP			D6 BCRP			D6 MRP			D6 YWHAZ			D6 ACTIN			N11 YWHAZ			N11 ACTIN					
I	N6 P-gP			N6 BCRP			N6 MRP			N6 YWHAZ			N6 ACTIN			D13 P-gp			D13 BCRP			D13 MRP		
J	D7 P-gP			D7 BCRP			D7 MRP			D7 YWHAZ			D7 ACTIN			D13 YWHAZ			D13 ACTIN					
K	N7 P-gp			N7 BCRP			N7 MRP			N7 YWHAZ			N7 ACTIN			N12 P-gp			N12 BCRP			N12 MRP		
L	D8 P-gp			D8 BCRP			D8 MRP			D8 YWHAZ			D8 ACTIN			N12 YWHAZ			N12 ACTIN					
M	N8 P-gp			N8 BCRP			N8 MRP			N8 YWHAZ			N8 ACTIN			D15 P-gp			D15 BCRP			D15 MRP		
N	Water RT P-gP			Water RT BCRP			Water RT MRP			Water RT YWHAZ			Water RT ACTIN			D15 YWHAZ			D15 ACTIN					
O	RT neg P-gP			RT neg BCRP			RT neg MRP			RT neg YWHAZ			RT neg ACTIN			N14 P-gp			N14 BCRP			N14 MRP		
P	Water-water P-gP			Water-water BCRP			Water-water MRP			Water-water YWHAZ			Water-water ACTIN			N14 YWHAZ			N14 ACTIN					

Table 5: Example PCR plate layout. Note all samples are run in triplicate and controls were run using water (in place of mastermix sample) and RT negative cDNA samples.

The PCR reaction was performed in the TaqMan 7900 HT PCR machine (Applied Biosystems) after spinning the plate in a centrifuge for 1 minute at 1000rpm. To run the PCR reaction the following was done: The SDS 2.4 programme was opened, new assay standard, 384 well plate selected. All wells with same colour detector – ACTB FAM for all the assays used for the above reactions, were selected and this was copied to the plate. Empty wells were highlighted and "omit wells" selected. The instrument tab was opened, sample volume set to 20uL and the realtime tab selected prior to highlighting the whole plate. The "connect to instrument" tab was pressed and the PCR plate was inserted into the machine prior to pressing "start run" tab. Changes to document were saved in the relevant folder. After the run had finished, data was extracted by highlighting all the wells, clicking "analyse" and then "results". Manual Ct was selected to adjust threshold accordingly (so line is just below the point where plots on the graph begin to flatten off). The image was saved and then export and data saved to the correct location.

mRNA quantification

The outcome data from the PCR reaction was analysed in the Applied Biosystems SDS Software with manual thresholds adjusted and auto baselines to calculate the Ct values

for each sample. Data was exported and analysis was performed in Microsoft Excel® and Ct values averaged (all samples were run in triplicate). The relative expression values were calculated normalising for the endogenous control (*YWHAZ*) and for the internal control sample (a non-diabetic sample which was run on every PCR plate).

GraphPad® Prism 6 was used to perform statistical analysis and draw figures. Normal distribution was tested using a Shapiro-Wilk normality test and differences between groups were tested using unpaired t-tests for continuous variables and chi-squared test for categorical variables. Data are presented as mean and standard deviation unless stipulated otherwise.

3.2.3 Western blotting method

Protein Extraction and Quantification

Prior to commencing, the blocks from the tissue lyser were placed in -80˚C freezer for approximately 5minutes to cool them and the centrifuge was set to 4˚C. 1 protease inhibitor (Roche) tablet was added to 10ml RIPA buffer (Sigma-Aldrich) and mixed by hand. Approximately 30mg of placental tissue was placed in a 2.0ml Eppendorf tube with a silver bead and 500uL RIPA buffer. The tissue was then homogenised in the cooled plates of the Qiagen Tissue Lyser at 25Hz for 3 minutes (run twice giving a total of 6 minutes) and then centrifuged at 4˚C at ≥10,000rpm for 10minutes and the supernatant extracted and aliquoted into 100uL volumes and stored at -80˚C.

Protein quantification was performed using Biorad DC protein assay solutions as per the manufacturer's instructions in RIPA buffer. The top standard (stock solution) had a concentration of 1.37mg/ml; dilution A was made with 20uL stock standard + 20uL RIPA buffer giving concentration of 0.685mg.ml; dilution B with 20uL of dilution A + 20uL RIPA buffer giving a concentration of 0.342mg/ml; dilution C with 20uL dilution B

+ 20ul RIPA buffer giving a concentration of 0.171mg/ml. 5uL of samples (in a 1 in 20 dilution[2]) and standards were added to the relevant wells in duplicate; an example plate layout is outlined below. 25uL of working reagent A (20µL reagent S to each 1ml reagent A needed for assay) was added to each well on the plate and then 200µL reagent B was added to each well using multichannel pipette. This was then incubated on the bench for 15minutes (timed) prior to quantification being performed using a Molecular Devices Thermomax microplate reader at 650nm-750nm and Softmax Pro software.

	1	2	3	4	5	6	7	8	9	10
A	1.37mg/ml		Sample D							
B	0.685mg/ml		Sample E							
C	0.342mg/ml		Sample F							
D	0.171mg/ml		Sample G							
E	BLANK		Sample H							
F	Sample A		Sample I							
G	Sample B		Sample J							
H	Sample C		Sample K							

Table 6: Example layout for protein quantification plate

The Lowry protocol was selected with 650nm wavelength LMI and automix performed for 10 seconds prior to plate reading according to the plate template. The results were then extracted. If any of the protein concentrations are higher than the top standard concentration then an "R" would be recorded on the results and a lower concentration of protein mix used and the test repeated.

[2] This dilution was found to be necessary as the protein solutions otherwise had a higher concentration than the top standard and protein quantification could not be performed.

This data was then exported to Microsoft Excel where calculations of the total protein concentration (averaged over the duplicates that had been run) were made and used to calculate the volume of supernatant required to give a 4mg/mL concentration of protein. A pooled sample of non-diabetic controls was made to create a cross-gel control.

Running the proteins through the gel

Protein samples were thawed on ice. LDS sample buffer and reducing agent (Invitrogen NuPage®) (2.5uL LDS sample buffer + 1uL reducing agent per sample) were mixed and 3.5uL added to each 0.2ml Eppendorf. The appropriate amount of protein and pure water (calculated as above to give 4mg/ml concentration and total volume of 6.5uL) were then added to each tube prior to vortexing for 5 seconds and then centrifuging for 1 minute at ≥10,000rpm . Samples were then placed in the G storm for 10minutes at 70˚C to denature.

Novex NuPage 4-12% bis-tris gels were used for running the samples in MOPS running buffer (50ml MOPS in 950ml de-ionised water (dH_20)) with 500uL antioxidant according to manufacturer's instructions. 10uL of SeeBlue Plus 2 Prestained standard (Nove, Invitrogen) was placed into the left-hand well of each gel to form a ladder, 10uL of the control sample (to allow inter-gel comparison) was placed in the next along well and then 10uL of each sample were placed in the subsequent wells, alternating protein samples from GDM and non-diabetic patients. The lid was then placed on the tank, ensuring correct placement for the leads and these were then connected to the energy pack. The gels were run for 1-hour 10minutes at a constant 180V.

Transfer step

Immobilon FL membrane (Merck Millipore) was used for protein transfer; 2 pieces were cut to 8x10cm (cut one corner at angle to allow orientation later) being sure to touch the membrane with only tweezers. 2 pieces of absorbent paper were cut to same size and these along with 2 sponges were placed in transfer buffer (800ml dH_2O, 100ml 10xTransfer buffer[3], 100ml methanol mixed) whilst the membrane was activated. 3 small plastic bowls were placed on the bench and enough methanol placed in the first, dH_2O in the second and transfer buffer in the third, all to depth of about 0.5cm. Holding the membrane with tweezers, it was first submerged in methanol for 30seconds, then dH_2O for 30 seconds and then placed in the transfer buffer until needed.

Setting up for transfer the cassettes were placed black side down in transfer buffer (in a large dish), the sponge added, followed by the paper, the gel (which has been carefully removed from its plastic casing, then the membrane (with the cut corner over the top of the ladder to orientate it) and then the final paper and sponge on top. A falcon tube was rolled on top to remove any bubbles prior to closing the cassette tight and placing it in the tank (which itself is sitting in a glass tray), black to black. An ice block was added to the tank and then the tank filled with transfer buffer. The lid was placed on the tank, leads correctly attached to energy pack and ice placed around the tank (in the glass tray) to maintain a cool temperature. The transfer was run at constant 100V for 1hour and 30minutes.

[3] 10 x Transfer Buffer = 24.2g Tris Base, 112.3g glycine, make up to 1L with dH_2O

Blocking step

For blocking, 5% Marvel milk solution (2.5g Marvel milk powder with 50ml TBST[4]) was placed in a small block box (same size as membrane) and the membrane removed from the cassette using tweezers and placed in the box (orientated so cut corner and standard ladder are on left-hand side) and left on a rocker to agitate for one hour at room temperature. The following primary antibodies[5] were applied in 10ml 5% milk solution: *MDR1/ABCB1* (P-gp) Rabbit monoclonal antibody (D3H1Q Cell signalling technology®) at a concentration of 1:1000 (10uL) and anti-alpha Tubulin mouse monoclonal antibody (AbCam®, ab80779) (this was used as an endogenous control) at a concentration of 1:5000 (2uL). The membrane was then incubated at 4˚C overnight, in a small plastic pouch with the primary antibodies, whilst being continuously agitated. The membrane was then washed in TBST for 5 minutes followed by TBS three times for 5 minutes each. Fluorescent LiCor secondary antibodies were applied in 10ml 5% milk solution both at a concentration of 1:10000: 1uL Donkey anti rabbit (P-gp) and 1uL Donkey anti mouse (α-Tubulin). This was then covered with foil (to minimise light exposure) and agitated at room temperature for one hour. Washing was repeated as above prior to being processed in a LiCor Odyssey® FC imaging system, adjusting the light intensity for each channel accordingly.

Protein Quantification

Analysis was performed using Image Studio Lite version 4.0 with protein quantity results extracted and transferred to Excel for processing to calculate relative

[4] TBST = 900ml dH2O + 100ml 10xTBS + 1ml Tween20
10xTBS = 12.11g Tris HCL + 87.66g NaCl, pH 7.6, make up to 1L with dH_20
[5] These primary antibodies were selected after trialling several different P-gp antibodies

concentrations of the transporter levels. Normal distribution was checked using a Shapiro-Wilk normality test and unpaired t-tests were performed.

3.3 RESULTS

Demographics of the placental samples used are outlined in table 7 and figure 7 below. They demonstrate that there was no significant difference in BMI across the groups of women from whom the placental samples were obtained.

	Gestational Diabetes Mellitus (n=36)	**Control Samples (n=36)**	**Statistical significance (p value)**
Gestation at delivery (weeks)	38.5 ± 2.4	39.1 ± 2.2	0.24
Maternal age	33 ± 6.4	31.7 ± 6.1	0.34
Parity	1.5 ± 1.4	0.9 ± 0.7	0.06
BMI	41.4 ± 8.3	41.0 ± 8.1	0.86
Smoker n(%)	5 (13.5)	2 (3)	0.50
Mode of delivery n and (%) - **Elective LSCS** - **Emergency LSCS** - **Spontaneous vaginal delivery** - **Instrumental delivery**	 24 (68) 3 (8) 7 (19) 2 (5)	 26 (71) 5 (14) 4 (11) 1 (3)	χ^2=0.24
Birth Weight (grams)	3620 ± 0.8	3630 ± 0.5	0.96
Ethnicity n (%) - **White** - **Other** - **Not documented**	 32 (89.2) 1 (2.7) 3 (8.1)	 26 (71.4) 0 10 (28.6)	
Treatment given for GDM n and n (%) - **Diet** - **Metformin** - **Metformin + insulin** - **Insulin** - **Not documented**	 9 (26) 15 (40) 10 (29) 1 (3) 1 (3)		

Table 7: demographics of women from whom placental samples were collected
The means, standard deviations and p values are given for the main confounding factors that could affect placental composition. This demonstrates that there is no significant difference between the group compositions. The different treatment modalities for the placental samples in the GDM group are also shown. Data are mean ± SD or n(%)

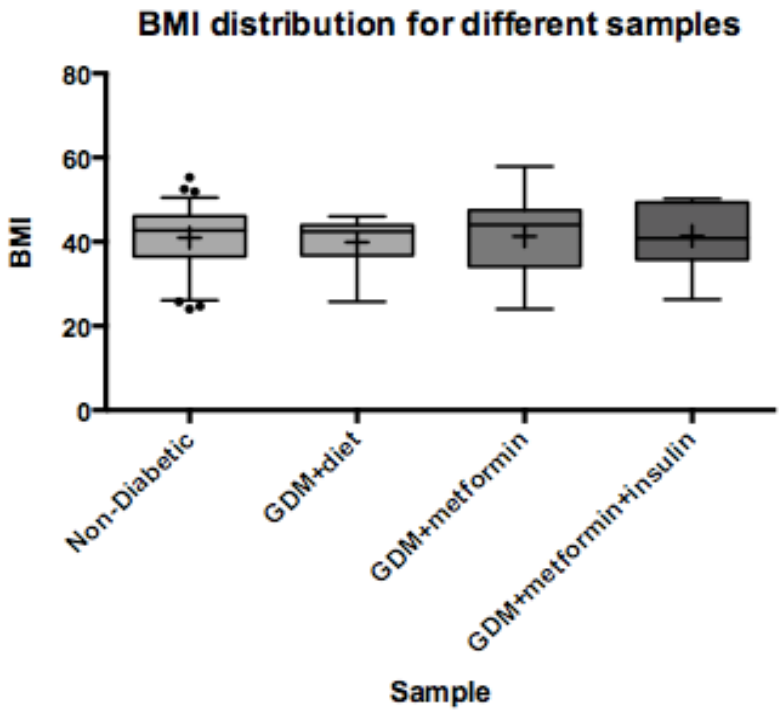


Figure 7: BMI for different sample groups in placental samples cohort
This figure demonstrates that there was no significant difference in BMI across any of the sample groups for the cohort of placental samples examined. The boxes represent quartiles enclosing 50% of the data, with the whiskers marking to the 10th and 90th centiles, the median is marked by a line and the mean by "+", outliers are marked by a " ● ".

When comparing mRNA levels of *ABC B1*, *ABC G2* and *ABC C3* between placental samples from women with GDM and those from non-diabetic patients there was no significant difference between the groups for any of the genes of interest (see figure 8 below).

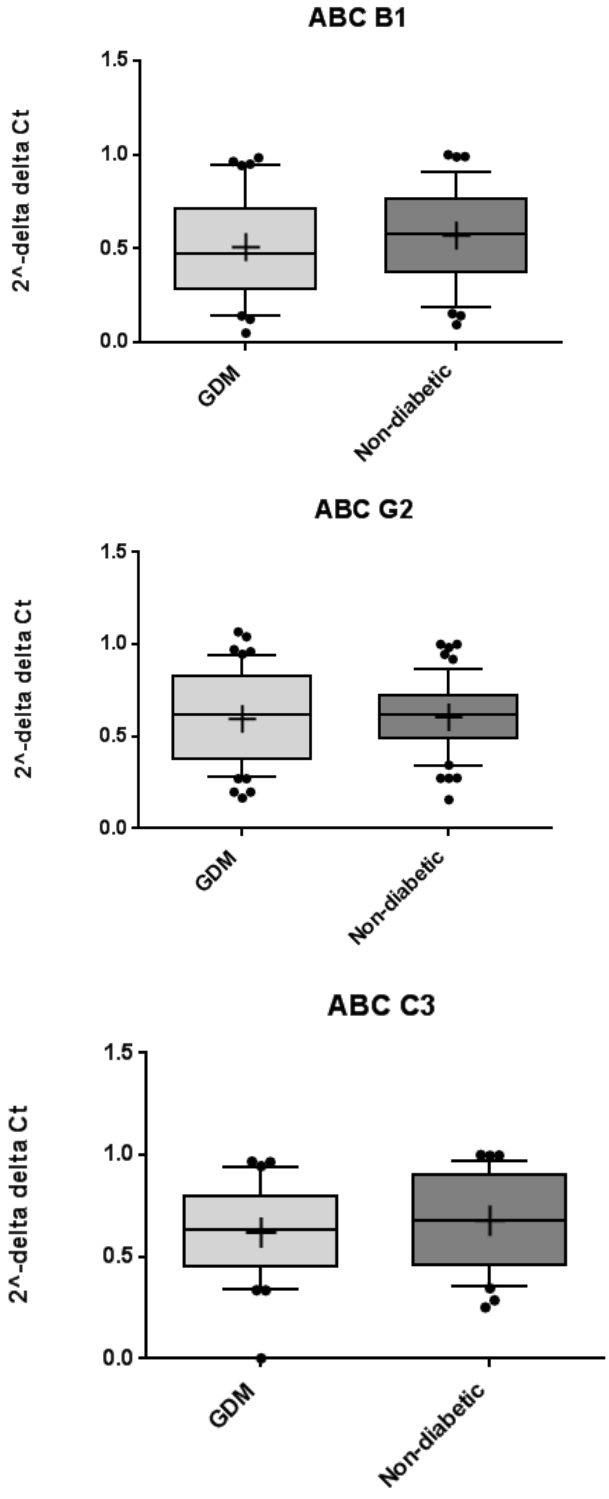


Figure 8: mRNA levels of ABC transporters in GDM and control samples
This figure demonstrates the mRNA levels of the three ABC transporters of interest (*ABC B1, ABC G2, ABC C3*) comparing between placentas from women with GDM and those without diabetes. The boxes represent quartiles enclosing 50% of the data, with the whiskers marking to the 10th and 90th centiles, the median is marked by a line and the mean by "+", outliers are marked by a " ●".

When the GDM group was broken down according to the treatment given to the patient, the *ABC B1* mRNA levels (see figure 9) were significantly lower in the metformin and insulin group compared to the placental samples from metformin alone treated GDM patients (0.40 ± 0.25 vs. 0.73 ± 0.41, p=0.026) and compared to the samples from non-diabetic patients (0.40 ± 0.25 vs. 0.63 ± 0.35, p= 0.04). This difference was not seen between the other treatment groups or for *ABC G2* and *ABC C3*.

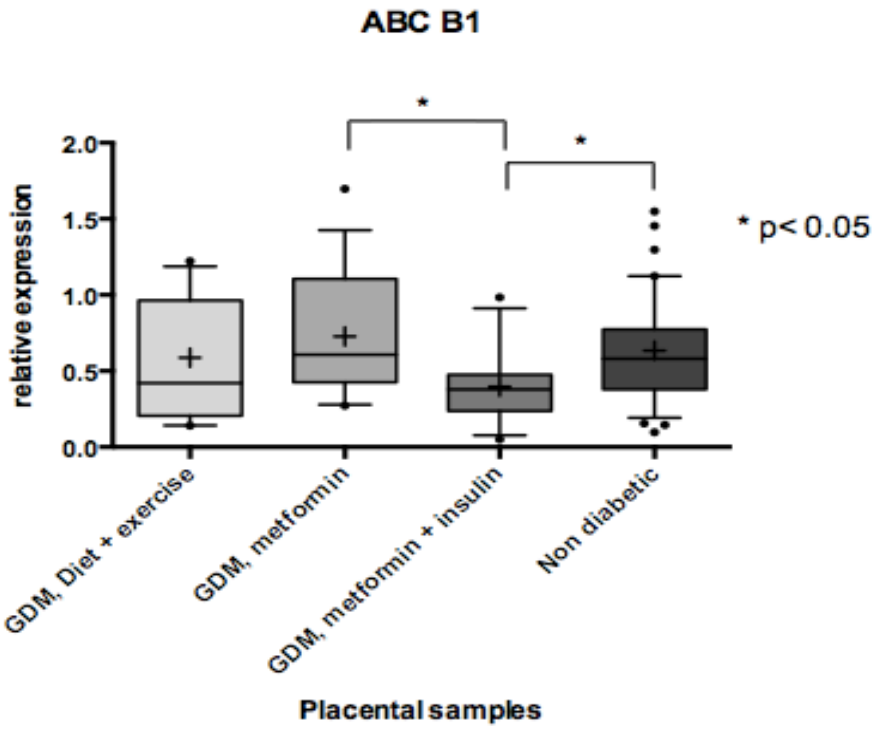
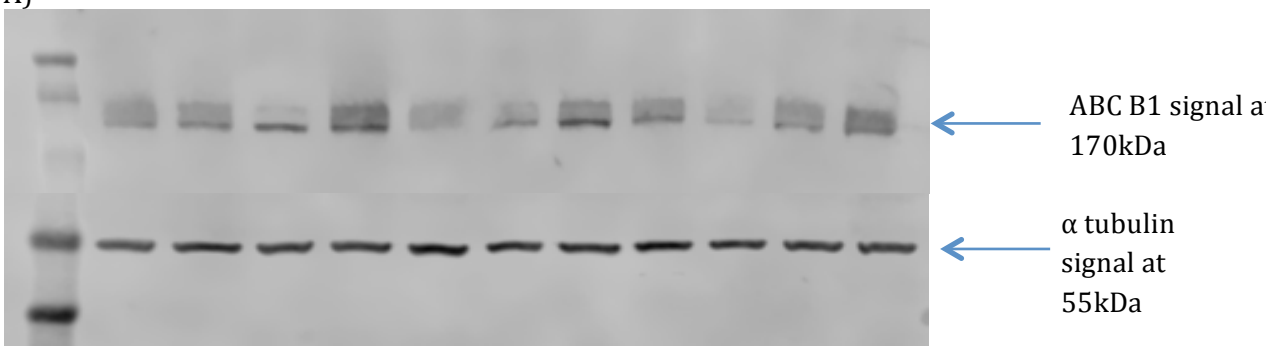


Figure 9: expression of *ABC B1* according to treatment of GDM and compared to non-diabetic controls
This figure demonstrates *ABC B1* expression, showing a reduction in mRNA expression in the placental samples from metformin and insulin treated GDM patients compared to those treated with metformin alone and compared to samples from non-diabetic patients. The boxes represent quartiles enclosing 50% of the data, with the whiskers marking to the 10th and 90th centiles, the median is marked by a line and the mean by "+", outliers are marked by a "●".

Following these findings these sample groups were compared using a western blot to see if this difference at mRNA translated across to a difference in protein level. Figure 10 shows the results of the comparison of protein level between the GDM on metformin, GDM on metformin + insulin placental samples compared to the non-

diabetic controls, demonstrating that the mRNA difference did not translate across to a difference in protein expression.

A)



B)

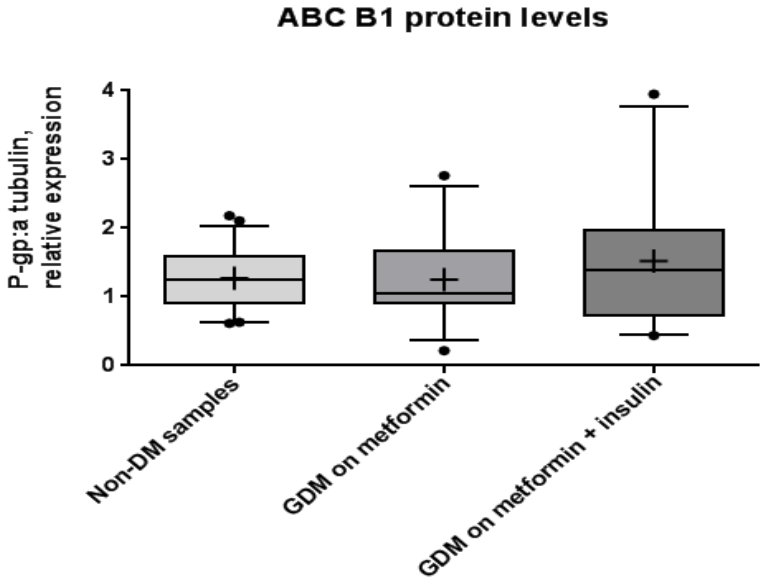


Figure 10: Protein levels of *P-gp (ABC B1 gene product)*

A) An example of the western blot obtained using the method described earlier. On the left is the standard ladder, the first sample from the left is the pooled non-diabetic control sample that was used as a control and then the samples alternate between non-diabetic and GDM.

B) This figure demonstrates the protein levels of P-gp between placental samples obtained from women with GDM treated with metformin and metformin + insulin and non-diabetic samples, showing no significant difference in levels between the groups. The boxes represent quartiles enclosing 50% of the data, with the whiskers marking to the 10th and 90th centiles, the median is marked by a line and the mean by "+", outliers are marked by a " ● ".

3.4 DISCUSSION

Anger et al demonstrated a greater level *of ABC B1* mRNA in a cohort of women with GDM treated with insulin compared to placental samples from non-diabetic women but were unable to identify any alteration in levels for the other ABC transporters. Despite the rising importance of OHAs in the treatment of GDM, there has been no examination of the levels of the ABC transporters in placental samples from women with GDM treated with OHAs and this lead to the development of the work outlined above.

A large cohort of placental samples (n=72) obtained from the ERTBB were examined, with GDM samples and healthy (non-diabetic) controls matched for BMI and gestation at delivery. As has been outlined above, there was no difference seen in the levels of the *ABC* transporters between placentas from GDM and non-diabetic women. However, if the GDM samples were broken down according to the treatment modalities, there was a significantly lower level of *ABC B1* at the mRNA level (although not the protein level) in the cohort of placentas from women with GDM treated with metformin and insulin. This was compared to both the cohort treated with metformin alone and the non-diabetic cohort. This finding is opposite to the hypothesis made prior to commencing the work as well as the findings from the Anger et al study; although the results were consistent between the two studies in that the difference in *ABC B1* mRNA levels was not translated to any changes in protein level. Earlier work by Anger et al using a rat model demonstrated a significant elevation in *ABC B1* in the placenta from rats with streptozotocin-induced GDM [109]. Interestingly, a cohort of the rats with GDM were also treated with insulin to treat hyperglycaemia and among this cohort, the elevation in *ABC B1* seen in the treated GDM cohort normalised back to levels approximating to those in the non-diabetic cohort. Whilst these findings did not translate across to the findings from their human study, it is suggestive that the insulin has resulted in a

decrease in expression of *ABC B1*, similar to that seen in the cohort of placentas from women with GDM treated with metformin + insulin from our work.

Another reason for the discrepancy between the Anger et al study and our result may be as a result of Anger failing to control for BMI between the GDM and non-GDM placental samples, this was controlled for in our samples (as can be seen in figure 7 above) with no significant difference between any of the treatment groups. When analysing the mRNA levels according to BMI there was no significant trend in expression of any of the genes of interest according to BMI.

The main difference between the cohorts of placental samples used in our study compared to those from the Anger study was the variance in treatment for GDM that the women had been on during their pregnancies. Whilst we looked at placental samples from women on diet alone, metformin and metformin and insulin, Anger only looked at samples from women treated with insulin alone. However, based upon Anger's findings, the hypothesis was that *ABC B1* levels would increase in placentas from women treated with insulin. If we were to extrapolate this, I would have expected to see this even in the cohort on metformin and insulin compared to those only on metformin however the opposite finding was seen.

Despite the differences between our work and that of Anger et al, we have provided novel work looking at the impact of GDM treated with OHAs on the levels of ABC transporters in the placenta. This study benefited from a large sample size and also the samples were collected after the completion of treatment (retrospective design); thereby ensuring that the treatment taken by the patients reflects those of patients in the real world and not confounded by improved treatment compliance whilst in a study.

The main limitation of this study is that there is no data on the glycaemic control that the women had during their pregnancies, such as HbA1c levels. This data is not obtained clinically in NHS Lothian and therefore is not available for the ERTBB. It could be inferred that those participants who required insulin as well as metformin may have had poorer glycaemic control at some stage of their pregnancy but this could have been at any stage and the addition of insulin then gave them better control.

In conclusion, this novel piece of work has provided evidence that *ABC B1, ABC G2 and ABC C3* placental transporters mRNA and protein levels are not altered by the disease process of GDM when compared to non-diabetic control samples. However, there does seem to be a reduction of *ABC B1* mRNA level among those samples obtained from women with GDM treated with metformin and insulin compared to those treated with metformin alone and those who were not diabetic, although this does not translate to a difference in protein level. These findings are reassuring in support of the use of metformin for the treatment of GDM as it suggests that the disease itself will not render the fetus susceptible to increased exposure of these xenobiotics used to treat the condition.

4. Effect of exposure to metformin and glibenclamide on placental *ABC* and *GLUT 1* transporters

As has been demonstrated in chapter 3, the administration of metformin in pregnancy does not appear to alter the expression of the ABC transporters in the placentas taken from women with GDM. However, glibenclamide is not a treatment currently used in NHS Lothian therefore there were no samples in the ERTBB to conduct this work on. It is also likely that over coming years the combination of two OHAs (such as metformin and glibenclamide) will be used to treat GDM; this is a well-recognised treatment for type 2 diabetes mellitus outwith pregnancy, known to delay or even avoid insulin therapy in a large proportion of patients. Therefore it would seem pertinent to examine the impact these individual treatments and the two in combination have on the ABC and GLUT 1 transporters of the placenta.

As a result I designed and conducted the following experiment to examine the impact of oral hypoglycaemic agents on the transporters:

- Placental explant culture in metformin and glibenclamide to assess whether exposure to these drugs alters the mRNA expression of the above mentioned ABC and GLUT 1 transporters.

GLUT 1 is the primary transporter responsible for the transfer of glucose to the fetal circulation, with a higher presence on the microvillus membrane compared to the basal membrane [110]. It has been hypothesised that this asymmetric distribution means that the transporters on the basal membrane act as the rate limiting step in glucose transfer to the fetus [111]. Interestingly, despite this, it has been shown that in placentas from diabetic pregnancies (pre-existing and GDM) *GLUT 1* is up-regulated on the basal membrane and not the microvillus membrane [112, 113]. This may explain

the increased incidence of fetal macrosomia even in diabetic pregnancies that are well controlled (HbA1c near normal); this is because an increased expression of *GLUT 1* will result in an increase in glucose transport to the fetus and subsequent hyperglycaemia and hyperinsulinaemia resulting in fetal macrosomia.

Knowing this, *GLUT 1* mRNA levels will also examined in these explant cultures, as an up regulation of *GLUT 1* as a result of the treatment would be an unwanted side effect.

Whilst it could be argued that the ABC transporters are preventing the drugs from accumulating in the placental circulation so thereby making any action of the drugs on the transporters unlikely; it is well known that these transporters are not 100% effective with evidence showing that whilst most of the medications get effluxed away from the fetal circulation, not all does, thereby allowing for the possibility of an interaction between the drugs and transporters.

4.1 Hypothesis

Exposure to metformin and glibenclamide does not alter the mRNA expression of ABC B1, ABC G2, ABC C3, GLUT 1 transporters.

4.2 Methods

Prior to arrival of the placental sample the 10x working solutions were prepared using the following quantities to get the concentrations required:

Metformin (10mM/ml) - 165.62mg/L:

- Make to 10ug/ml: 10uL in 1.66ml complete culture medium (CCM)[6]
- Make to 50ug/ml: 50uL in 1.66ml CCM

[6] This was made with RPMI 1640, penstrep and fetal calf serum with an approximate glucose concentration of 11mmol/L.

Glyburide (10mM/ml) – 4940mg/L:

- First dilute neat glyburide to 1in10 dilution.
    - 100uL glyburide into 0.9ml DMSO = 1mM/ml = 494mg/L
- Make to 1ug/ml (x10 conc): 5uL 1in10 glyburide into 2.47ml CCM
- Make to 5ug/ml (x10 conc): 20uL 1in10 glyburide into 1.9mls CCM

Fresh placental samples were obtained from normal, non-diabetic women with a BMI between 20 and 30m/kg^2 undergoing an elective caesarean section delivery at Simpson's Centre for Reproductive Health Edinburgh (again these samples were obtained under the umbrella of the ERTBB). Following delivery of the placenta, an 8x10cm full-thickness specimen was obtained and placed in DPBS at room temperature. It was then immediately transferred to the laboratory where the specimen was inspected, washed in DPBS to remove excess blood and explants measuring 70-100mg were obtained from the maternal-facing surface of the placenta, attempting to avoid blood vessels/granulation tissue (4 samples representative of those cut were weighed to check the weight).

The following culture mediums were made with these drug concentrations chosen because they represent the average and maximum serum concentrations in women treated for GDM, as reported in the literature:

- Complete Culture medium (CCM) control
- Metformin in CCM at concentration 1ug/ml
- Metformin in CCM at concentration 5ug/ml
- Glibenclamide in CCM at concentration of 0.1ug/ml
- Glibenclamide in CCM at concentration of 0.5ug/ml

- Metformin 1ug/ml + Glibenclamide 0.1ug/ml
- Metformin 1ug/ml + Glibenclamide 0.5 ug/ml
- Metformin 5ug/ml + Glibenclamide 0.1ug/ml
- Metformin 5ug/ml + Glibenclamide 0.5ug/ml

24 well Corning® Costar® cell culture plates were then set up to contain the appropriate concentration of each drug and CCM in the respective wells with each condition repeated 4 times and then the placental sample added to each well. The culture plates were then incubated in 37˚C, 5% Co_2 for 24hrs.

Cultures were performed eight times (eight different placentas), with four repeats for each placenta in each culture condition, giving an *n* of 32. For 2 of the placental samples, additional explants were run in the above conditions but these ones were removed after 2 hours.

Following the culture period, 50-100uL supernatant was removed and placed in 0.2ml Eppendorf tubes and placed in -20°C freezer. Placental samples were then removed from the supernatant, blotted to remove excess fluid and snap frozen on dry ice prior to storage at -80˚C. RT qPCR was later performed on these samples, analysing the expression of *ABC B1, ABC G2, ABC C3 and GLUT* transporters (same method used as in chapter 3).

Samples of supernatant were tested at 2hrs and 24hrs of culture for lactate levels by ELISA to assess whether there was any significant change during the culture time that may have suggested that the tissue was no longer viable at 24hours. Data was checked for normal distribution and one way ANOVA analysis was performed.

mRNA quantification

RT qPCR results were analysed in the Applied Biosystems SDS Software with manual thresholds adjusted and auto baselines to calculate the Ct values for each sample. Data was exported and analysis was performed in Microsoft Excel® and Ct values averaged (all samples were run in triplicate). The relative expression values were calculated normalising for the endogenous control (*YWHAZ*) and for an internal control sample. GraphPad® Prism 6 was used to perform statistical analysis and draw figures. Normal distribution was tested using a Shapiro-Wilk normality test and differences between groups were tested initially using an ANOVA analysis (if normally distributed) or Kruskal-Wallis test (for non-normally distributed results) and then individual unpaired t-tests for continuous variables and chi-squared test for categorical variables between the groups. Data are presented as mean and standard deviation unless stipulated otherwise.

4.3 RESULTS

As can be seen from figure 11 below, culturing the placental samples in metformin, glibenclamide or the combined treatment did not result in any significant difference in mRNA levels of *ABC B1, ABC G2, ABC C3* or *GLUT* 1 transporters.

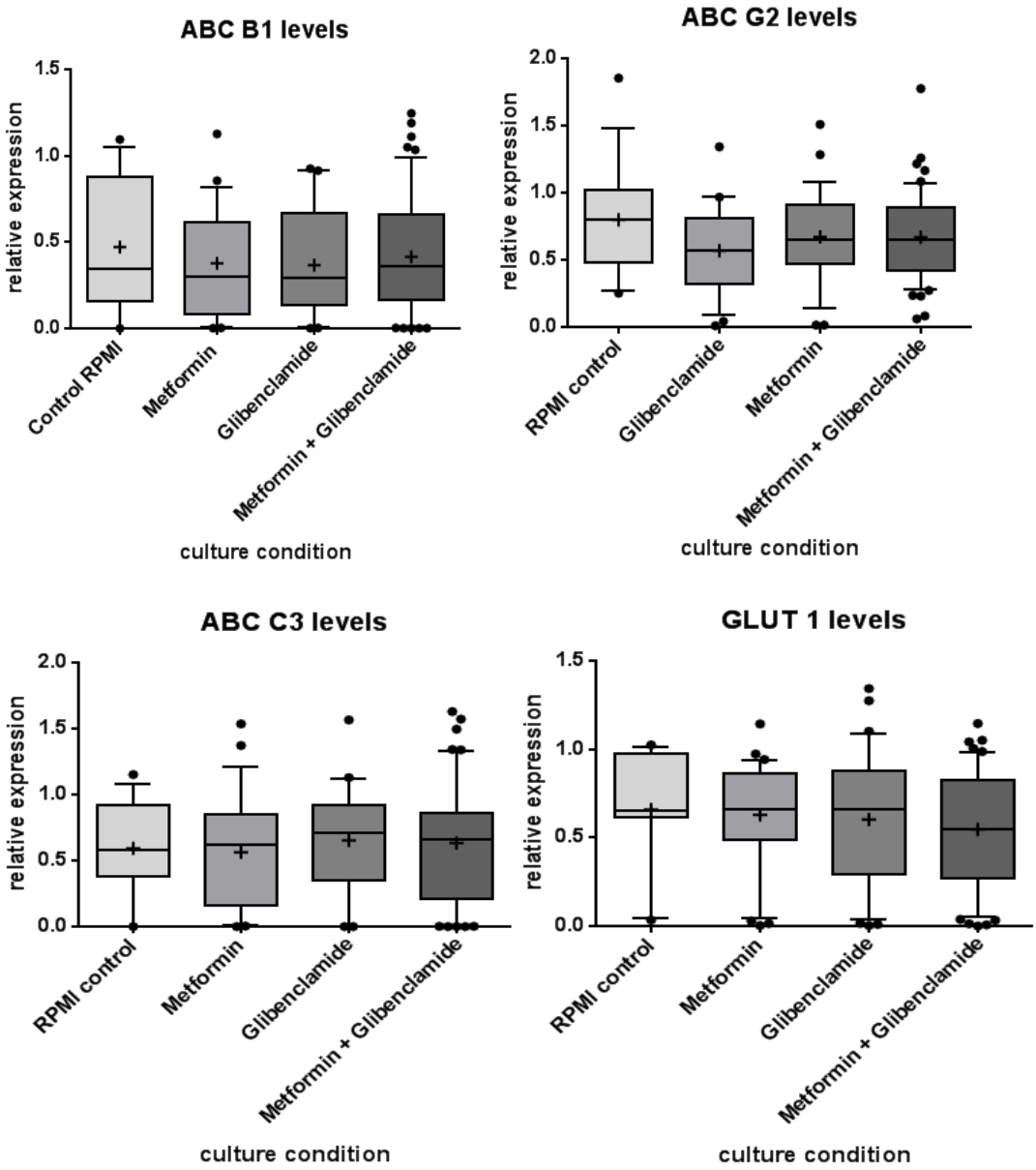


Figure 11: Relative expression of ABC and GLUT 1 transporters in placental explant following 24hr culture

These figures represent the findings for the placental samples cultured in the above conditions. Following statistical analysis, there was no significant difference between the mRNA levels of these transporters between any of the culture conditions. The boxes represent quartiles enclosing 50% of the data, with the whiskers marking to the 10th and 90th centiles, the median is marked by a line and the mean by "+", outliers are marked by a " ● ".

On two of the placental samples, the cultures were run for 2hrs and 24hrs to see if this resulted in any difference in transporter levels in the different conditions. These findings are demonstrated in figure 12 below.

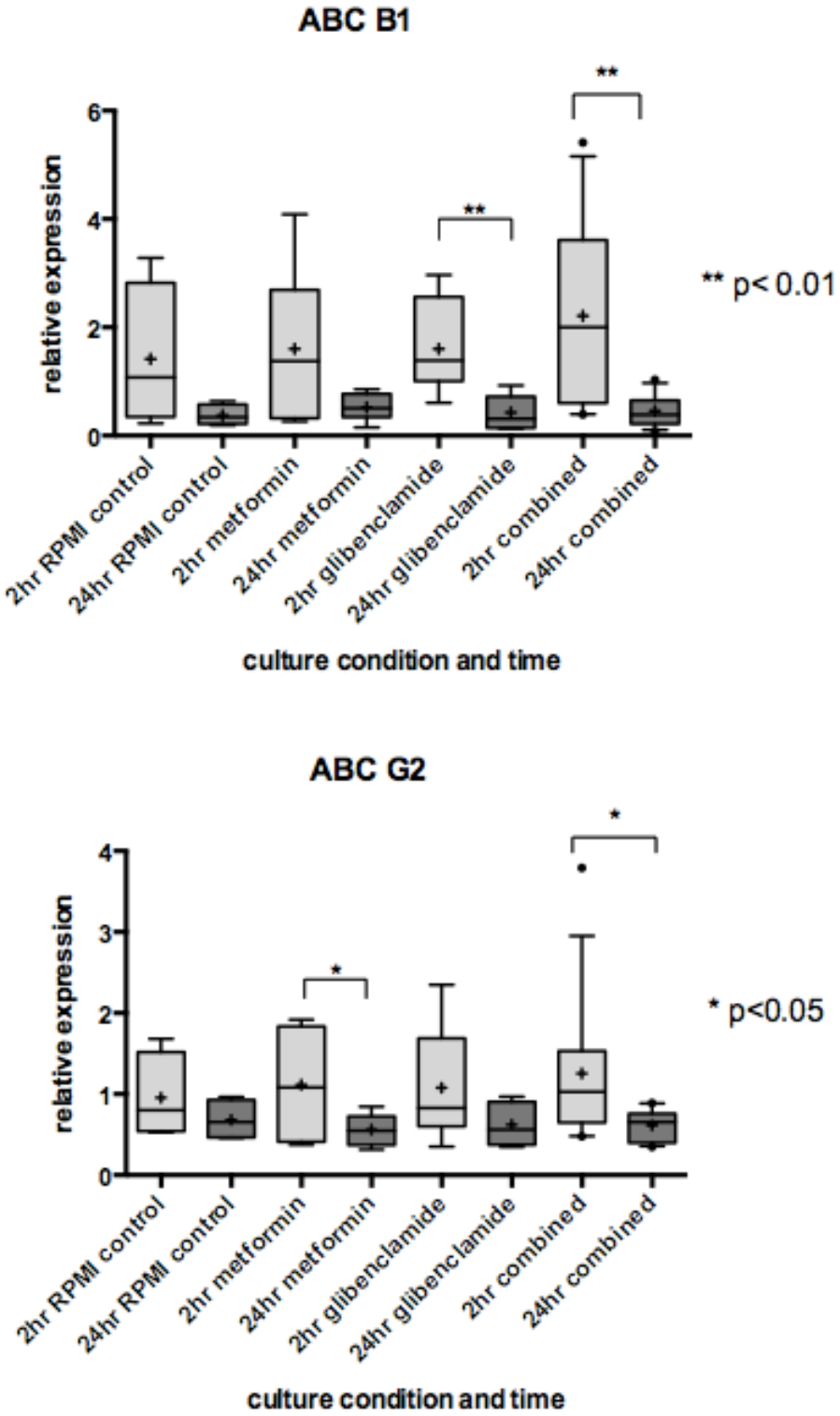


Figure 12: Expression of ABC B1 and ABC G2 and GLUT 1 in placental explant following culture at 2hrs and 24hrs

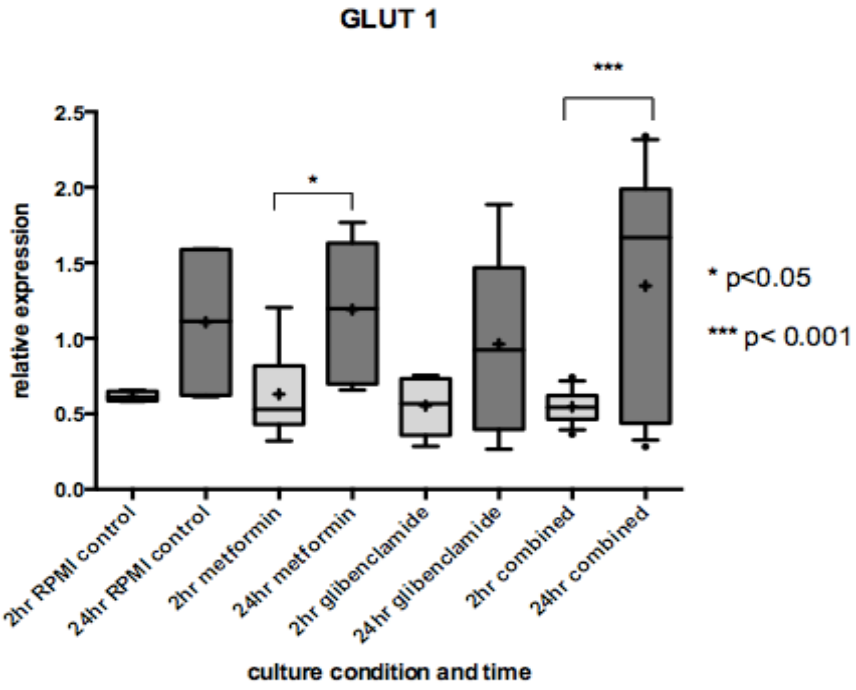


Figure 12: Expression of ABC B1 and ABC G2 and GLUT 1 in placental explant following culture at 2hrs and 24hrs

These figures represent the mRNA levels for *ABC B1* and *ABC G2* and *GLUT 1* for the placental samples cultured in the above conditions showing the difference between 2hrs and 24hrs of culture. The boxes represent quartiles enclosing 50% of the data, with the whiskers marking to the 10th and 90th centiles, the median is marked by a line and the mean by "+", outliers are marked by a "●".

When looking at the levels of *ABC B1 and G2,* there was a significant reduction in mRNA levels at 24hours compared to 2 hours; a one-way ANOVA of all the culture groups gave a $p<0.01$ for *ABC B1* and $p<0.05$ for *ABC G2*. This difference was not seen with the *ABC C3* levels and the inverse (i.e. an increase in expression from 2 hours to 24 hours) was seen in the *GLUT 1* levels (Kruskal-Wallis test giving $p=0.02$). This may simply represent the effect of time and raise the possibility that the placental tissue was denaturing over the 24hour culture time and therefore the RNA yield was lower although this does not explain the increase in GLUT 1 levels.

Importantly, when analysis was performed looking at the mRNA levels of the individual transporters after only two hours of culture, the findings were the same as that of the

24hour culture, i.e. there was no significant difference in mRNA level for any of the genes of interest between the different culture conditions.

Samples of the supernatant were collected and analysed for lactate level at 2hrs and 24hrs of culture as a measure of tissue viability. Figure 13 demonstrates a significant increase in lactate level between 2hrs of culture and 24hrs of culture (6.05 ± 0.74 vs. 6.93 ± 0.275, p< 0.001).

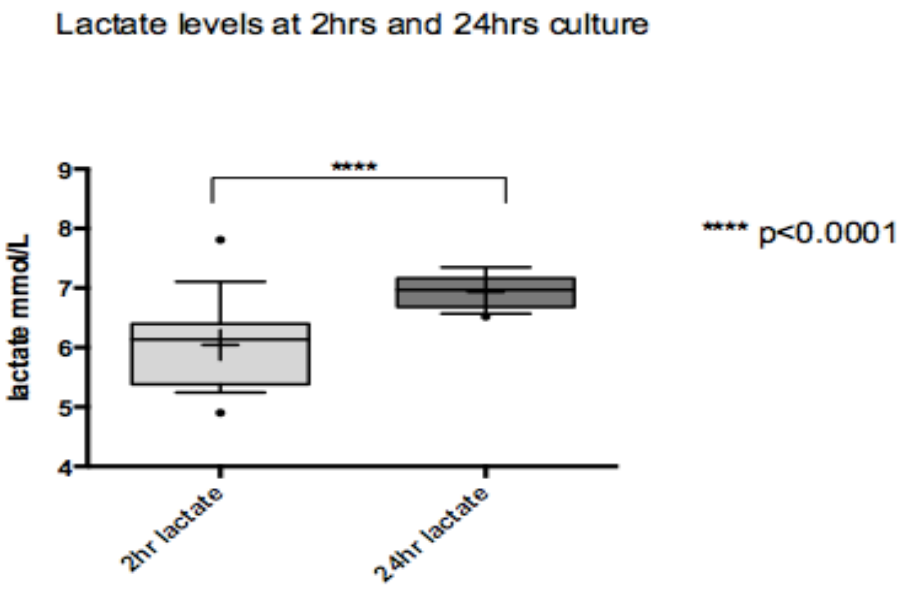


Figure 13: Lactate concentration (mmol/L) in culture supernatant at 2hrs and 24hrs culture
This demonstrates a significant difference in the lactate concentrations in the supernatant at 2hrs and 24hrs of culture. The boxes represent quartiles enclosing 50% of the data, with the whiskers marking to the 10th and 90th centiles, the median is marked by a line and the mean by "+", outliers are marked by a " ●".

4.4 Discussion

In chapter 3 above, we demonstrated that the disease process of GDM does not alter the expression of the ABC transporters in the placenta and looked at the impact of treatment with metformin and metformin + insulin. We then extended the work to look at the impact of the two main groups of oral hypoglycaemic agents (metformin and glibenclamide) would have on normal placental tissue (i.e. placental explants obtained

from healthy non-diabetic women) as this was something that had not be explored before.

Placental explants were obtained from eight healthy, non-diabetic women with a BMI between 22-29m/kg^2 following elective caesarean sections and each culture condition was repeated four times for each placenta (giving an *n* of 32). Examining the mRNA levels of the *ABC* and *GLUT 1* transporters after 24 hours of culture failed to demonstrate any change in levels among any of the culture conditions.

Eight of the cultures ran for 24 hours under the conditions described above. For two of the cultures, extra samples were run and these were removed after two hours to see whether this had any impact on mRNA yield and transporter mRNA levels as it was noticed that the mRNA yield on the samples after 24 hours of culture (assessed on the Nanodrop 1000) was much lower than that seen in the previous work. As can be seen in figure 14 above, there was a significant difference in the relative expression of mRNA for some of the different transporters seen between the two hours and 24 hours of culture.

24 hours culture time was initially chosen as it was felt that this would be long enough to allow any mRNA modification to occur if it was going to happen and also still be within the time of tissue viability demonstrated in several papers, although it was noted that there was a degree of tissue degeneration during the initial 24 hours of culture [114, 115].

Samples of supernatant were obtained at two hours and 24 hours of culture from each of the culture conditions and lactate levels were assessed as a measure of tissue viability as this has been demonstrated in several papers as an indicator of tissue viability [45, 107].

The tissue culture work has demonstrated that culture of healthy term placental tissue in metformin, glibenclamide and a combination of both at concentrations representative of those seen clinically does not alter the mRNA levels of the *ABC* transporters or *GLUT 1* transporters examined.

However, there are some clear limitations to this piece of work. Firstly, the difference in mRNA levels between 2hours and 24hours is concerning and given that only two of the cultures had samples removed at 2 hours it is difficult to clearly assess the relevance of these findings. The lactate levels seen suggest that some tissue degeneration has occurred between two and 24 hours of culture, although it may simply be a reflection of the metabolic activity of the tissue during this culture time. A study looking at placental explant viability between seven and 24hours demonstrated pronounced alteration in the cultured tissue between seven and 24hours with marked apoptosis of the trophoblast [116]. This is clearly demonstrated in the figure below.

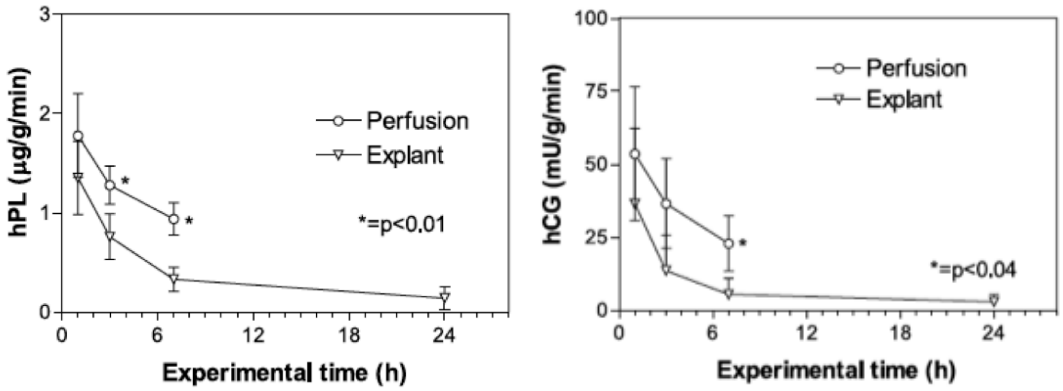


Figure 14: accumulation of hPL and hCG during seven to 24hours of placental culture. Values given as mean ± SD. [116].

The culture medium used was made from RPMI that has a glucose concentration of 11mmol/L. This is significantly hyperglycaemic and could well have resulted in an alteration in the mRNA levels of the transporters that could have masked any changes made by the drugs. It has also been suggested that hyperglycaemic conditions can

induce apoptosis of culture tissue, however this was shown not to be the case in a paper examining tissue viability over 24 hours [116]. It would have been ideal to repeat these tissue cultures under normoglycaemic conditions to explore any effect of the hyperglycaemia, which has previously been shown to alter the expression of *GLUT 1* and also *ABC G2*, however this was not possible due to time limitations.

Another aspect that could be considered in further work is the gestation of the placental samples used. All the placentas used in this work were term placentas however, as mentioned in chapter 2, levels of the ABC transporters (especially *ABC B1*) alter with gestation. It would therefore seem pertinent to perform this work on second trimester placentas [7] as well to see if this altered the findings. However, the difficulty of obtaining 2nd trimester placentas (especially those from healthy "normal" pregnancies) would need to be taken into consideration.

In conclusion; this work has demonstrated that placental explant culture with the two oral hypoglycaemic agents used in clinical practice does not appear to alter the mRNA expression of *ABC B1, G2 and C3* and *GLUT 1* over a 24 hour period. However, there are limitations to this work that have been discussed above. It would be ideal to be able to examine the mRNA and protein levels of these transporters in placentas from women with GDM who were treated with these drugs (both individually and in combination) during their pregnancy, giving a more accurate measure of the true impact of these drugs.

[7] First trimester placentas would not need to be tested as GDM tends to develop from the second trimester and treatment before then with OHAs is uncommon.

Conclusion

The incidence of GDM is increasing with rising levels of obesity and a recent change in diagnostic criteria. This increased incidence and knowledge of the importance of treating GDM to minimise the consequences of uncontrolled hyperglycaemia have driven the search for appropriate treatments forwards. Over the last 8 years it has become increasingly common to treat GDM with OHA's such as metformin and glibenclamide and whilst there is a large body of evidence demonstrating the clinical safety and efficacy of such treatments, little attention has been given to the mechanisms in place to protect the fetus from exposure to such mediations and other xenobiotics.

Growing evidence has identified the presence of the *ABC* transporters in the placenta and documented the role they play in protecting the fetus from unwanted substances including xenobiotics. As outlined in the literature review above, there are three *ABC* transporters of particular importance with regard to the treatment of GDM with OHAs: *ABC B1, ABC G2 and ABC C3*.

The work in this thesis has provided important evidence that GDM does not alter the very transporters that protect the fetus from unwanted exposure to the OHA treatments given to mothers for GDM. The culture work performed has also demonstrated that in the *in vitro* setting, there does not appear to be an alteration in the expression of *ABC B1, ABC G2, ABC C3* and *GLUT 1* following exposure of placental explants to metformin, glibenclamide or both at levels representative of those seen clinically in patients on the treatment.

The next steps following this work should be to conduct a clinical trial looking at the effectiveness and safety of using combined oral hypoglycaemic agents (metformin + glibenclamide) for the treatment of GDM. As well as providing safety and clinical data,

this would also provide a cohort of placental samples from women who received the combined treatment during pregnancy allowing a more realistic examination of mRNA and protein levels of the ABC transporters.

Performing placental perfusion experiments looking at the action of the ABC transporters in the presence of metformin and glibenclamide would be a pertinent next step to ensure that combined treatment does not result in competition for the binding sites and hence an accumulation of one or other drug in the fetal circulation.

Another factor that has not been addressed in this work is the presence of BCRP variants, especially C421A, which can reduce BCRP expression due to a reduction in transcriptional efficacy. The C421A variant is particularly prevalent among Caucasians, Chinese and Japanese populations (10-30%) [69]. Given that glibenclamide is predominately effluxed from the fetal circulation via BCRP it would seem sensible to assess whether transport of glibenclamide is different between placentas from women with and without the C421A variant of BCRP.

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